The Journal of Laboratory and Clinical Medicine

VOLUME 28
OCTOBER, 1942—DECEMBER, 1943

ST. LOUIS
THE C. V. MOSBY COMPANY
1943

Printed in the
United States of America

Press of
The C. V. Mosby Company
St. Louis

The Journal of Laboratory and Clinical Medicine

Vol. 28 October, 1942 No. 1

CLINICAL AND EXPERIMENTAL

HEART DISEASE AND LIVER FUNCTION*

Mitchell Bernstein, M.D., Edward B. Le Winn, M.D., and Samuel Simkins, M.D., Philadelphia, Pa.

HERETOFORE, studies of the relationship of hepatic function to heart disease have been based solely upon clinical observations.[1-5] A review of the literature fails to disclose any studies correlating liver function and cardiac disease, as determined by precise methods. In the present report we have attempted to study this relationship by means of the bromsulfalein retention test and by the measurement of the velocity of blood flow and the venous pressure.

MATERIAL AND METHODS

A group of 59 patients, free from hepatobiliary disease was studied. There were 29 men and 30 women, ranging in age from 19 to 79 years. These patients were studied clinically and quantitatively as to the status of the circulation and liver. For the purpose of this investigation the patients were divided as follows:

A. *Control Group:* Patients free from any demonstrable evidence of heart disease. Five patients with uncomplicated hyperthyroidism were included in this group.

B. *Cardiac Group:* These patients, evidencing definite heart disease, were for practical purposes[6] subdivided on a functional basis as follows:

1. Fully compensated heart disease.
2. Mild cardiac decompensation, evidenced by exertional dyspnea and cyanosis.
3. Moderate cardiac decompensation, evidenced by dyspnea, cyanosis, and pulmonary congestion, the last named being demonstrable clinically or by roentgen-ray examination.

---

*From the Medical Service of Mitchell Bernstein, M.D., Jewish Hospital, Philadelphia.
Received for publication, April 11, 1942.

4. Marked cardiac decompensation, evidenced by symptoms and signs of groups 2 and 3 plus hepatomegaly, edema, or ascites. Three patients were listed in more than one group in accordance with their changing functional cardiac status.

In the group of cardiac patients were 14 with hypertensive cardiovascular disease, 16 with arteriosclerotic heart disease, 5 with myocardial infarction, 6 with rheumatic heart disease, 2 with subacute bacterial endocarditis, and one with adhesive pericarditis.

The circulation was studied quantitatively by means of the venous pressure and the magnesium sulfate and ether circulation time tests. The venous pressure was measured directly by the use of an L-tube, calibrated in millimeters and centimeters, to which was attached an 18 gauge needle. Both tube and needle were thoroughly flushed with 2.5 per cent sodium citrate solution. The patient was in recumbent position, except when prevented by extreme cardiac insufficiency. The arm was abducted to a 45 degree angle and supported on a pillow which was so arranged as to place the antecubital fossa 5 cm. posterior to the level of the fourth sternochondral junction. A tourniquet was applied to the arm, and the needle was inserted into a large antecubital vein. The patient was cautioned to lie and breathe as quietly as possible. After the fluctuations of the venous column of blood subsided, the pressure was read directly in centimeters of blood. With the needle left in situ 5 c.c. of warm 10 per cent magnesium sulfate solution were injected as rapidly as possible and the end point was determined, giving the arm-to-tongue circulation time. One minute later 5 minims of ether mixed with 5 minims of normal saline solution were injected and the end point was determined, giving the arm-to-lung circulation time.

By means of the bromsulfalein retention test an index of the liver function was obtained. A dose of 5 mg. of bromsulfalein per kilogram of body weight was employed. The bromsulfalein determination followed or preceded the study of the circulation as closely as possible, usually within one or two hours. After injection of the dye, readings were made from specimens of blood collected at intervals of five, fifteen, and thirty minutes.

A total of 70 sets of circulation times, venous pressure, and bromsulfalein determinations were performed in the 59 patients.

Criteria.—The following values were used as normals in determining the status of the circulation:

Venous pressure: 3 to 12 cm. of blood.

Arm-to-tongue circulation time (magnesium sulfate circulation time[6]) eight to fourteen seconds.

Arm-to-lung circulation time (ether circulation time) three to eight seconds.

Lung-to-tongue circulation time (magnesium sulfate circulation time minus ether circulation time) up to eight seconds.

Failure of the left side of the heart was evidenced by a reading of lung-to-tongue circulation time greater than eight seconds. Failure of the right side of the heart was evidenced by a reading of ether circulation time greater than eight seconds and/or by a reading of the venous pressure over 12 cm. of blood.

Bromsulfalein retention of 10 per cent was considered as the upper limits of normal.

RESULTS

A. *Control Group.* Fifteen sets of determinations were performed on 15 noncardiac patients. In 14 patients the bromsulfalein liver function test was normal, whereas one patient showed an abnormal bromsulfalein retention without demonstrable cause.

B. *Cardiac Group.*—In 10 patients comprising group 1 (fully compensated heart disease), 12 of 13 determinations made showed agreement and one determination showed disagreement between the cardiac status and the liver function. Group 2, consisting of 10 patients, disclosed 11 determinations in which there was agreement and 3 with disagreement. Of 14 determinations made on 14 patients in group 3, 6 determinations showed the cardiac status and the liver function tests to be in agreement and 8 showed disagreement. In group 4, consisting of 13 patients, 11 of 14 determinations showed agreement between the cardiac status and liver function, whereas 3 showed disagreement.

Three cases are worthy of special mention. One patient, a 63-year-old man with acute myocardial infarction, at first disclosed failure of the right and left sides of the heart, as evidenced by the circulation time. There was marked cyanosis which persisted throughout a prolonged convalescence. Although the magnesium sulfate circulation time decreased steadily from 25.4 seconds to 14.4 seconds within a period of twenty-two days, with a corresponding drop in ether circulation time from 16.0 to 7.6 seconds, the parallel bromsulfalein determination ranged from 40 per cent to 28 per cent retention. It is very probable that the anoxemia, as evidenced by the cyanosis, may have been a causal factor in the continuance of the abnormal bromsulfalein retention. Another patient, a 60-year-old woman with decompensation due to hypertensive cardiovascular disease, showed exceptional parallelism between the liver function and the cardiac status as the state of compensation progressively improved, both clinically and by the method of measurement described above. A third patient, a 52-year-old woman with rheumatic aortic stenosis complicated[s] by hyperthyroidism, showed slight abnormal bromsulfalein retention (12 per cent) with moderate failure of the right side of the heart; venous pressure 14.8 cm.; arm-to-tongue (magnesium sulfate) circulation time 15.8 seconds; and arm-to-lung (ether) circulation time 10.6 seconds. It is to be noted in the foregoing case that the circulation times would undoubtedly have been much higher were it not for the marked tendency of the hyperthyroidism to decrease the circulation time.

DISCUSSION

Our studies indicate that the bromsulfalein excretion is normal in the absence of heart failure (control and compensated heart disease groups). In mild degrees of cardiac failure (group 2) the bromsulfalein excretion is impaired in close parallelism with the status of the circulation. Incidentally, it is worthy of mention that in mild degrees of cardiac decompensation the circulation times and venous pressure may fall within normal limits in a fairly high percentage of patients, a fact previously brought out by the authors.[6]

It is noteworthy that in patients with definite pulmonary congestion (group 3) the correlation of hepatic function with the condition of the circula-

TABLE I

CLINICAL CLASSIFICATION OF CASES	NUMBER OF PATIENTS	NUMBER OF TESTS	A. BROMSULFALEIN EXCRETION	B. CARDIAC STATUS AS DETERMINED BY CIRCULATION TIMES AND VENOUS PRESSURE				CORRELATION OF TESTS‡ A AND B			
				NO HEART FAILURE	HEART FAILURE PRESENT			AGREEMENT		DISAGREEMENT	
					RIGHT	LEFT	BOTH	NUMBER	%	NUMBER	%
Controls*	15	15	Normal	14	0	0	0	14	93.5	1	6.5
			Abnormal	1	0	0	0				
1. Compensated heart disease	10	13	Normal	12	0	0	0	12	92.3	1	7.7
			Abnormal	1	0	0	0				
2. Mild cardiac failure	10	14	Normal	6	1	0	0	11	78.5	3	21.5
			Abnormal	2	1	2	2				
3. Moderate cardiac failure†	14	14	Normal	0	1	5	2	6	42.8	8	57.2
			Abnormal	0	1	5	0				
4. Severe cardiac failure	13	14	Normal	0	1	1	1	11	78.5	3	21.5
			Abnormal	0	0	5	6				

*Includes 5 cases of hyperthyroidism uncomplicated by cardiac disease.
†Includes one case of hyperthyroidism.
‡A test consists of bromsulfalein determination and circulation times and venous pressure.
§There were 59 patients of whom 3 were listed in more than one group in accordance with their changing functional cardiac status.

2. Day, T. D., and Armstrong, T. G.: Fibrosis of the Liver in Heart Failure, J. Path. & Bact. 50: 221, 1940.
3. Boland, E. W., and Willius, F. A.: Changes in the Liver Produced by Chronic Passive Congestion, Arch. Int. Med. 62: 723, 1938.
4. Kahler, H.: Clinical Diagnosis of So-called Intermediary Congestion of the Liver in Cardiac Decompensation, Wien. Arch. f. inn. Med. 33: 167, 1937.
5. Jolliffe, N.: Liver Function in Congestive Heart Failure, J. Clin. Investigation 8: 419, 1930.
6. Bernstein, Mitchell, and Simkins, Samuel: The Use of Magnesium Sulfate in the Measurement of Circulation Time, Am. Heart J. 17: 218, 1939.
7. Best, C. H., and Taylor, N. B.: The Physiological Basis of Medical Practice, ed. 2, Baltimore, 1939, Williams & Wilkins Co., page 467 ff.
8. Bernstein, Mitchell, and Simkins, Samuel: Aortic Stenosis Complicated by Hyperthyroidism, M. Record 155: 250, 1942.

1321 Spruce Street

A SIMPLIFIED BEDSIDE TEST FOR LATENT JAUNDICE*

Alan Leslie, M.D., New York, N. Y.

An ordinary, thin, 3 inch rubber band is the only apparatus required for this test, the technique of which is as follows: The rubber band is placed about the patient's upper arm, drawn taut mesially away from the skin, and then released. A typical "triple response" of Lewis[1] then takes place. We are not concerned with either the red reaction or the flare. A wheal (third phase of the "triple response") appears in five to fifteen minutes. The surrounding skin is then stretched in order to blanch the area, which is the site of the erythematous flare. This may be done advantageously by pressure over a glass slide. The colors of the wheal and of the surrounding skin are compared in either day or artificial light, the former being preferred. A positive test for jaundice is characterized by yellow coloration of the wheal.

The physiologic principles underlying the test have been well known for considerable time. In 1931 Klein[2] demonstrated that in cases of jaundice wheals produced by the intracutaneous injection of histamine were darker yellow than the surrounding skin. He further stated that when the bilirubin content of the blood was raised to levels insufficient to produce clinically detectable jaundice, histamine wheals showed yellow coloration. In 1936, Brodribb and Cullinan[3] reported quantitative studies of this method. They injected 0.1 mg. of histamine in 1 minim of sterile solution intracutaneously in the upper arm or back. They were able to detect a yellow coloration of the wheal in cases of biliary obstruction or hepatic damage when the blood bilirubin was 0.5 mg. or more per 100 c.c. In cases of hemolytic jaundice positive tests were not seen unless the blood bilirubin concentration was at least 1.1 to 1.4 mg. per 100 c.c. These authors mentioned the possibility of positive tests in carotinemia and allied diseases in which there is increased pigment in the blood.

The wheal of the "triple response" of Lewis, which is due perhaps to liberated histamine or "H-substance," is physiologically indistinguishable from

*From the Medical Service of Dr. George Baehr, the Mount Sinai Hospital.
Received for publication, February 9, 1942.

wheals produced by histamine injected intracutaneously. Any brisk superficial localized trauma may be substituted for the rubber band method of raising the wheal, but because of its simplicity and convenience, this latter procedure is specifically recommended.

REFERENCES

1. Lewis, T.: The Blood Vessels of the Human Skin and Their Responses, London, 1927, Shaw & Sons.
2. Klein, O.: Über die Anreicherung von Bilirubin und intravenös injizierter Farbstoffe im Bereiche der durch intradermale Histamininjektion erzeugten Hautquaddeln, Med. Klin. 27: 625, 1931.
3. Brodribb, H. S., and Cullinan, E. R.: A Simple Test for Latent Jaundice, Lancet 1: 1237, 1936.

OBSERVATIONS ON ADDITIONAL INSTANCES OF A SUPERNORMAL PHASE IN THE HUMAN HEART*

Howard B. Burchell, M.D., Rochester, Minn.

In 1924 Lewis and Master[1] reported a case of heart block in which complete dissociation of the auricles and ventricles was interrupted by occasional conducted beats. It was noted that in order for conduction from auricle to ventricle to occur, the P wave had to fall in a definite time period after the preceding QRS complex of the idioventricular rhythm. Cases of this type are not rare, and an example (Case 1) has been chosen from two which I have encountered.

REPORT OF CASES

Case 1.—The patient was a man 73 years of age who suffered from attacks of syncope. Except for bradycardia and generalized senescent changes, physical examination gave negative results. An electrocardiogram (Fig. 1) revealed an idioventricular rhythm with a cycle of two seconds, and occasional interruption or interference of the cycle by premature ventricular complexes which followed a P wave with a constant P-R interval. When the P waves which were followed by ventricular complexes were charted (Fig. 2), it was seen that they fell between 0.56 and 0.88 second after onset of the preceding QRS complex; P waves falling outside this phase were not conducted.

Comment.—Lewis and Master explained this phenomenon by the presence of a supernormal phase of conductivity similar to a biologic phenomenon observed by Adrian and Lucas[2] in 1912 and described in greater detail by Adrian[3] in 1921. These observers demonstrated that in injured, excitable tissue there was a short period during recovery from a previous stimulus in which the tissue became hypersensitive to new stimuli. In 1925 Ashman[4] observed the existence of the supernormal phase of conductivity in the hearts of turtles, in which varying degrees of auriculoventricular block had been produced. The work of Hoff and Nahum[5] in 1938 indicated that a supernormal phase of excitability could be present in relatively normal mammalian ventricular muscle.

A second type of cardiac mechanism exhibiting evidence of the supernormal phase is much more rare and is that of paroxysmal heart block occurring con-

*From the Division of Medicine, the Mayo Clinic, Rochester.
Received for publication, March 4, 1942.

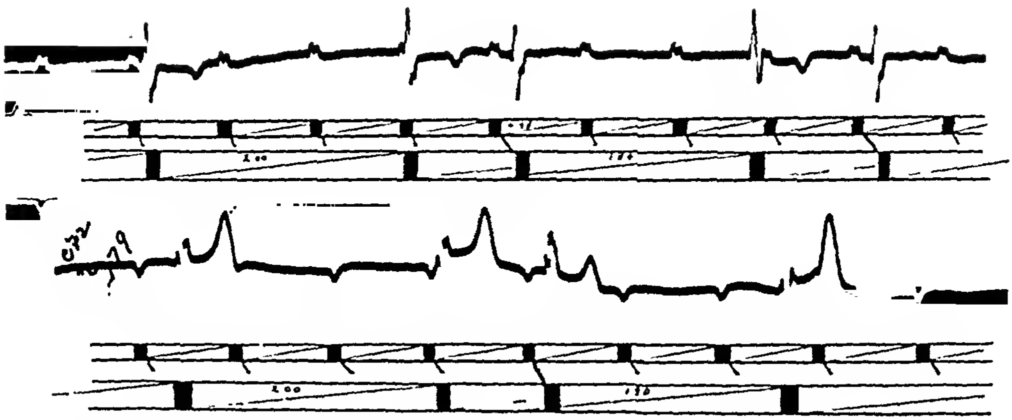


Fig. 1.—Complete heart block except for occasional auriculoventricular conduction. Two conducted beats are shown in the upper tracing (Lead II) and one in the lower tracing (Lead CF_2). The ventricular complex of the conducted beat has a different shape from the QRS complex of the idioventricular rhythm. It is also interesting to note that the returning cycle of idioventricular rhythm is shorter than might be expected.

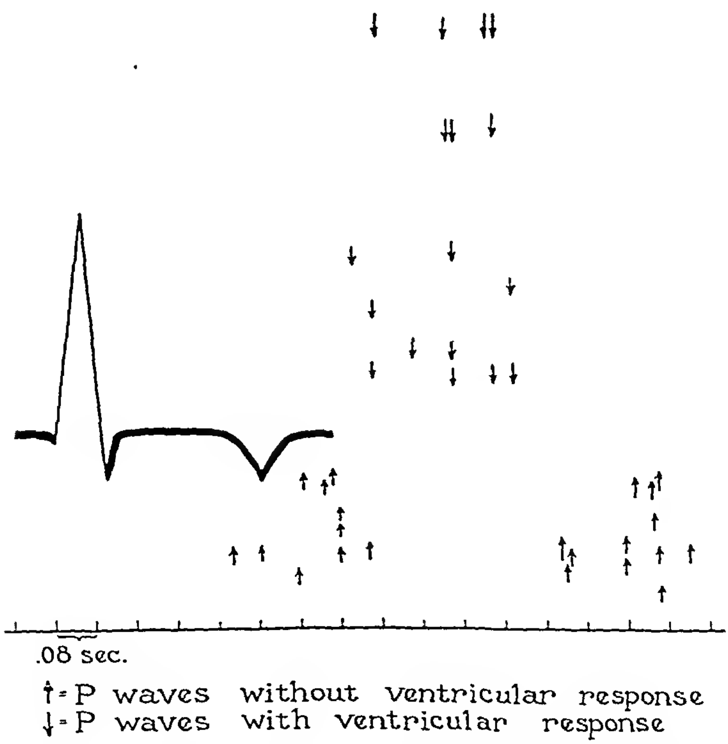


Fig. 2.—The positions of all the P waves that were followed by auriculoventricular conduction are compared with the positions of P waves associated with auriculoventricular block. There is a slight overlapping of two groups at the beginning of the time phase of favored conductivity, but not at the end of this phase. The P-R interval of the conducted beats is not charted but was normal and the same for all conducted beats.

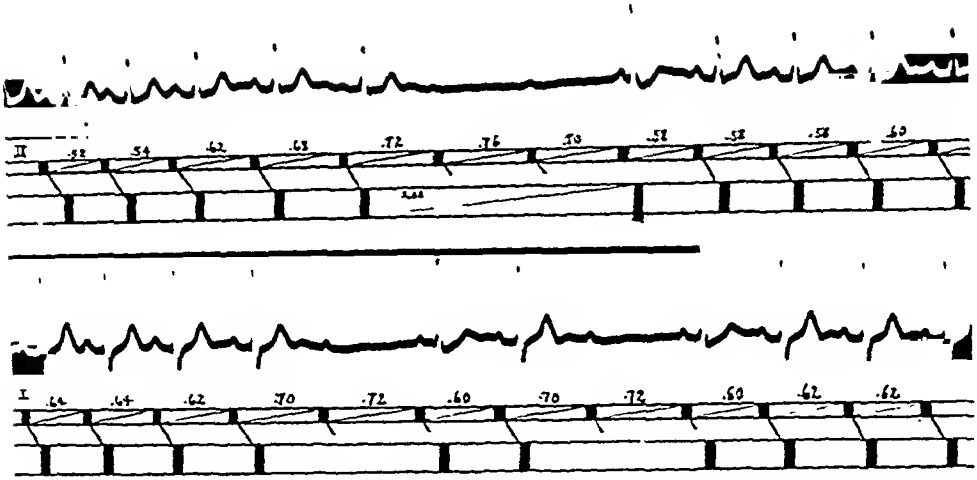


Fig. 3.—Paroxysms of complete auriculoventricular block following auricular slowing and resumption of auriculoventricular conductivity only when a P wave has fallen in a definite time relationship after an idioventricular beat are demonstrated. The phenomenon may be seen once in the upper tracing and twice in the lower. The P-P intervals are given in hundredths of seconds.

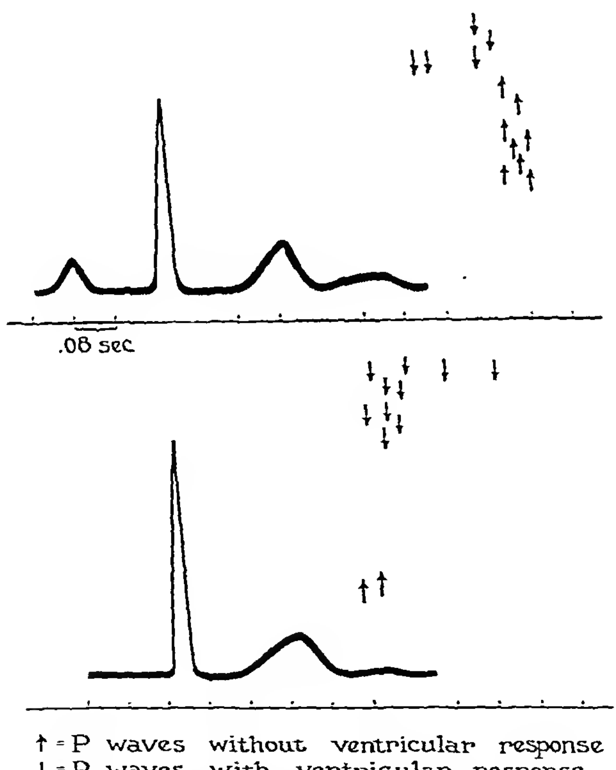


Fig. 4.—Upper portion demonstrates the position of P waves, always followed by auriculoventricular conduction and the maintenance of normal rhythm, and the position of P waves followed by auriculoventricular block. In the original tracing numerous P waves were present, but as they all had the same grouping, many were omitted in this figure as their numbers added nothing to a smaller representative group. In the lower portion of the chart P waves which followed an idioventricular beat and were associated with auriculoventricular conduction and the resumption of normal rhythm may be seen. Except for one possible exception P waves outside this time relationship were not associated with auriculoventricular conduction.

comitantly with auricular slowing. The electrocardiographic records obtained in Case 2 are similar to those obtained in a case reported by Wilson and Herrmann[6] and described later in detail by Ashman and Herrmann,[7] and to those in a case reported by Kline, Conn, and Rosenbaum.[8]

CASE 2.—The patient, a man 29 years of age, registered at the Mayo Clinic in February, 1940, and related a history of frequent attacks of syncope of two years' duration. It was noted on physical examination that the fainting spells were associated with ventricular asystole and that attacks could be reproduced by pressure on either of the carotid sinuses. There was a rather distant, harsh, aortic systolic murmur, and the heart was slightly enlarged by roentgenographic examination. History of rheumatic fever was not obtained.

On electrocardiographic study it was noted that complete auriculoventricular dissociation was dependent on auricular slowing. Auriculoventricular conduction was never re-established until one or more idioventricular beats had occurred, and the P wave of the first beat to be conducted had to have a definite time relationship to the preceding QRS complex. The tracings reproduced in Fig. 3 are representative of numerous records obtained, both in spontaneous attacks of auriculoventricular dissociation and in attacks induced by pressure on a carotid sinus. In the upper tracing reproduced in Fig. 3 it may be seen that there is a gradual slowing of the auricular rate, the cycle increasing from 0.52 to 0.76 second, with a gradual displacement of the P wave away from the preceding complex. It was noted that as soon as the P wave falls away from the U wave phase of the tracing, conduction to the ventricle no longer occurred. In the lower tracing of the same figure the same phenomenon occurs twice. It may be pointed out again that there was no resumption of the normal rhythm until an idioventricular beat has occurred, that is, an idioventricular beat had to sensitize the conducting tissues from below before an auricular impulse could be conducted from above. When the P waves were charted (Fig. 4), it was found that when they fell more than 0.68 second after the preceding QRS complex auriculoventricular dissociation occurred. With return of auriculoventricular conduction the P waves fell between 0.40 and 0.65 second after the preceding QRS complex except for the one exception charted. That a vagal influence on the conduction mechanism is unlikely, is indicated by the fact that temporary resumption of conduction may occur while the auricular rate is still slow. An explanation dependent on an increased blood supply to the bundle following a period of block does not prove satisfactory, since the time of conducted beats is independent of the periods of ventricular standstill. The outstanding difference between this case and the usual case of Adams-Stokes' attacks dependent on paroxysmal heart block is that in the latter, heart block usually is preceded by an increase in the auricular rate and failure of recovery in the conducting tissues.

Since the patient had frequent attacks of syncope, digitalis was administered in the hope that it might produce permanent auriculoventricular block and relieve the symptoms. It was found, however, that digitalis caused complete block only when symptoms of mild intoxication from digitalis were present. Administration of digitalis, therefore, was discontinued. After the patient returned home, the symptoms soon disappeared entirely, and he was able to do his routine work on the farm.

The patient returned to the clinic two years later, in February, 1942. It was found that he had complete auriculoventricular dissociation with a ventricular rate of 40. Subcutaneous administration of 1 c.c. of epinephrine (1:1,000) or 1/50 grain (0.0013 Gm.) of atropine did not have any effect in changing the complete block. His general condition at this time was excellent, although the aortic systolic murmur was louder, a faint diastolic murmur was present at the left of the sternum and the heart had increased slightly in size. During a continued period of auscultation the first heart sound occasionally had the accentuated loudness characteristic in certain cases of complete heart block.

SUMMARY

The presence of the supernormal period in the human heart has been used to explain cases of interference dissociation in which the auricles are beating

more rapidly than the ventricles, cases of paroxysmal heart block in which cessation and resumption of auriculoventricular conduction have a phasic dependency on the immediately preceding electrical events and cases of possible parasystolic rhythm. An example is given of each of the first two types of mechanism which are best explained by the existence of a supernormal period in excitability of the conduction tissues. Case 2 is considered of some importance, as it is another instance of the undoubtedly rare cases in which the supernormal period has played a role in the maintenance of normal sinus rhythm. Following establishment of complete block, the patient has remained in relatively good health for two years.

REFERENCES

1. Lewis, Thomas, and Master, A. M.: Supernormal Recovery Phase, Illustrated by Two Clinical Cases of Heart-block, Heart 11: 371, 1924.
2. Adrian, E. D., and Lucas, Keith: On the Summation of Propagated Disturbances in Nerve and Muscle, J. Physiol. 44: 68, 1912.
3. Adrian, E. D.: The Recovery Process of Excitable Tissues, J. Physiol. 55: 193, 1921.
4. Ashman, Richard: Conductivity in Compressed Cardiac Muscle. II. A Supernormal Phase in Conductivity in the Compressed Auricular Muscle of the Turtle Heart, Am. J. Physiol. 74: 140, 1925.
5. Hoff, H. E., and Nahum, L. H.: Supernormal Period in Mammalian Ventricle, Am. J. Physiol. 124: 591, 1938.
6. Wilson, F. N., and Herrmann, G. R.: Some Unusual Disturbances of the Mechanism of the Heart Beat, Arch. Int. Med. 31: 923, 1923.
7. Ashman, Richard, and Herrmann, G. R.: A Supernormal Phase of Conduction and a Recovery Curve for the Human Junctional Tissues, Am. Heart J. 1: 594, 1926.
8. Kline, E. M., Conn, J. W., and Rosenbaum, F. F.: Variations in A-V and V-A Conduction Dependent Upon Time Relation of Auricular and Ventricular Systole: Supernormal Phase, Am. Heart J. 17: 524, 1939.

PERCUTANEOUS TYPHOID PROPHYLAXIS*

ABRAHAM GELPERIN, M.D., M.P.H., AND DALE KESSLER, M.D.
CINCINNATI, OHIO

PERIODIC typhoid prophylaxis is necessary if there is continuous possibility of contact with the typhoid bacillus. Reinoculation by the intradermal route presents a technique that has promise of being the method of choice.[1] Since large numbers of persons are sometimes involved, it was deemed advisable to study the percutaneous route of administering antigenic stimulation in an attempt to simplify the procedure further.

It has been shown that many diverse substances are absorbed through the skin.[2, 3] In previous investigations huge doses of bacilli were utilized—as much as 100 mg. of bacilli by weight with an emulsion base.[4] Since such enormous amounts of antigen would be impossible of production, doses were used which seemed likely to be both antigenic and feasible of manufacture.

The typhoid strain utilized was isolated in 1926 at the Cincinnati General Hospital. Its Vi content is unknown. Standard vaccine was prepared, two billion bacilli to the cubic centimeter. The production of "H" antibodies was studied, since a measure of protective antibodies was not possible; and the production of "O" antibodies following intradermal inoculation was found to be

*From the Cincinnati Health Department, Cincinnati.
Received for publication, March 19, 1942.

TABLE I

GROUP	PATIENT	INITIAL TITER PRIMARY INOCULATION	RESPONSE TO PRIMARY STIMULATION SECOND STIMULATION	RESPONSE TO SECOND STIMULATION (INTRACUTANEOUS)	RESPONSE TO SECOND STIMULATION (PERCUTANEOUS)	INITIAL TITER PERCUTANEOUS RESTIMULATION	RESPONSE TO RESTIMULATION
		2/23/41	3/6/41	3/15/41	3/17/41	8/9/41	8/25/41
A (Control)	1	0	20	20		–	–
	2	0	320	640		40	40
	3	0	320	320		–	–
	4	0	320	640		–	–
	5	0	160	320		–	–
	6	0	320	640		0	20
	7	10	320	640		–	–
B (Disintegrated bacilli)	1	0	160		40	–	–
	2	0	40		10	0	0
	3	0	1,280		1,280	80	80
	4	10	40		40	20	20
	5	0	40		40	10	40
	6	10	10		10	0	0
	7	0	160		320	0	0
	8	0	40		20	–	–
	9	0	1,280		1,280	0	–
C (Whole bacilli)	1	0	640		320	–	–
	2	0	160		320	10	80
	3	0	160		80	0	0
	4	40	1,280		1,280	–	–
	5	0	40		40	–	–
	6	0	80		40	20	40
	7	0	40		10	–	–
	8	0	20		20	0	0
Total	24	24	24	7	17	13	13

practically nil by Van Gelder.[5] Our titration of "O" antibody gave the same result. Our first attempt at percutaneous stimulation utilized 20 billion typhoid bacilli a day for three consecutive days; the second employed a single dose of 200 billion bacilli.

Twenty-four patients of the Hamilton County Chronic Disease Hospital, who had no history of either typhoid fever or prophylaxis, were divided into three groups. Initially each patient received an intracutaneous primary antigenic stimulation of 0.1 c.c. of the prepared vaccine. The control group, A, was reinoculated fourteen days later with 0.1 c.c. intracutaneously. Group B at the same time vigorously massaged an emulsion of 20 billion disintegrated (alternate freezing and melting) bacilli into the skin of the forearm, and group C an equal dose of whole bacilli. The emulsions were applied to alternate forearms for three consecutive days. The base was Jergens lotion, and each 0.2 c.c. contained the required dose.

Nine days after the termination of the secondary antigenic stimulations, blood sera titrations revealed equivocal results in groups B and C, and increases in 5 of 7 persons in group A. Six months later 13 patients were retested, 2 from group A, 7 from group B, and 4 from group C. (The other patients were not available.) The initial serum titrations showed marked losses in all. Each patient vigorously massaged an emulsion of olive oil and whole bacilli into the forearm; each 0.2 c.c. dose contained 200 billion bacilli. Sixteen days later serum titrations revealed no significant increases in titer.

With the technique employed, no appreciable typhoid "H" antibody was stimulated by percutaneous vaccination.

REFERENCES

1. Siler, J. F., and co-workers: Immunization to Typhoid Fever, Baltimore, 1941, The Johns Hopkins Press.
2. Eller, J. J., and Wolff, S.: Permeability and Absorptivity of the Skin, Arch. Dermat. & Syph. 40: 900, 1940.
3. Ishikawa, R.: Immunological Studies on the Function of the Skin. III. On the Percutaneous Immuno-therapy, Mitt. a. d. med. Akad. zu Kioto 25: 900, 1939.
4. Saito, S.: Studien über einige Immunisierungsmethoden mittels Typhusvaccine; Antikörpererzeugung im Blute durch perkutane Applikation von Typhusvaccine, Acta dermat. 34: 1, 1939.
5. Van Gelder, D. W., and Fisher, S.: Intradermal Immunization. III. Typhoid Fever, Am. J. Dis. Child. 62: 933, 1941.

ACQUIRED DRUG RESISTANCE IN THE HEMOLYTIC STREPTOCOCCUS*

Morgan Cutts, M.D., and Albert V. Troppoli, Sc.M.
Providence, R. I.

MAC LEOD and Daddi,[1] and subsequently Lowell and co-workers[2] showed that some strains of pneumococci, previously susceptible to the action of sulfapyridine, can be rendered "resistant" by growing them in gradually increasing concentrations of the drug in vitro. Recently Hadley and Hadley[3] reported somewhat similar results in the case of a group A hemolytic streptococcus. Using increasing concentrations of sulfanilamide and an incubator temperature of 40° C., they were able to show an "adaptation" of the organism from 2.5 to 10 mg. per 100 c.c. drug concentration. They also noted a change from the mucoid to the smooth phase coincidentally with this adaptation. The present work was planned to investigate the production of drug resistance in vitro in the case of the hemolytic streptococcus, the association of phase transformations, and the duration and stability of the resulting modified strains.

The organism used throughout these experiments was a group A hemolytic streptococcus, freshly isolated from a case of acute pharyngitis. Culture media included plain blood agar plates without added peptone and a meat infusion broth of pH 6.8 likewise without added peptone. At one point in the experiments 20 per cent ascitic fluid was added to the standard broth to produce an enriched medium. The varying concentrations of the drug were obtained by adding the proper amounts of stock solutions of 50 or 100 mg. per 100 c.c. of sulfanilamide in broth.

In describing the various phases of the strains studied we have followed closely the outline of Dawson, Hobby, and Olmstead.[4]

At the outset considerable difficulty was encountered in selecting a suitable organism, for most strains whether freshly isolated or from stock cultures were little affected by concentrations up to 50 mg. per 100 c.c. of sulfanilamide in broth even when a small amount of inoculum was used. Finally the one used, GA, was found on repeated tests to show no growth in concentrations above 7.5 mg. per 100 c.c. sulfanilamide in broth. Morphologically, this organism closely resembled the other strains previously discarded. It formed small rounded colonies on blood agar whitish to reflected light with milky centers and no mucoid border and with a smooth margin when examined under low power. It formed homogeneous suspensions in saline and was not virulent for mice. The lethal dose was between 1 and 2 ml. of a twenty-four-hour broth culture. No capsules could be demonstrated. It resembled the typical "smooth" colony described by other workers. During the course of subsequent experiments it remained essentially stable, and only during the latter

*From the Charles V. Chapin Hospital, Providence.
Received for publication, April 13, 1942.

half did it show some tendency toward the M-S form, as evidenced by slightly increased size and flatness of the colony on blood agar, and by the occasional development of a mucoid "fringe."

Over a period of two weeks GA was subjected to growth in increasing concentrations of sulfanilamide in broth at a temperature of 37.5° C. This was begun at a concentration of 2 mg. per 100 c.c. and carried up by daily subcultures to a concentration of 100 mg. per 100 c.c. The resulting strain labeled GAR after being grown for several transfers in plain broth was tested for drug susceptibility at the same time as the parent strain GA which had meanwhile been carried in plain broth. The inoculum was small, i.e., one standard loopful of a twenty-four-hour broth culture was mixed with 5 c.c. of broth and a loopful of this mixture was used for the test. The results appear in Table I.

TABLE I

INITIAL COMPARISON OF DRUG RESISTANT AND ORIGINAL STRAIN

DRUG CONCENTRATION		0	5	7.5	10	15	20	25	35	50	75	100
GA	Broth	##	#	0	0	0	0	0	0	0	0	0
	Agar*	##	#	0	0	0	0	0	0	0	0	0
GAR	Broth	##	##	##	##	##	##	##	##	##	#	0
	Agar*	##	##	##	##	##	##	##	##	##	##	0

*Subculture from the corresponding broth tube after forty-eight hours. ##, Full growth: #, scant growth; 0, no growth.

The broth tubes were read at forty-eight hours, the agar plates at twenty-four hours.

As is evident a marked degree of drug resistance had been produced in GAR. Coincidentally with this, the phase of the organism had been changed, for GAR showed definite differences from the parent strain GA with regard to the colonies on blood agar plates. Comparing the two, GAR was smaller, had a finely stippled surface, and showed some roughening of the border, especially after forty-eight hours. GAR also showed some tendency to spontaneous clumping in saline suspensions as compared to none in GA. From this we inferred that GAR was in an intermediate stage between smooth and rough, an S-R form, while GA had remained smooth. This difference persisted and was even somewhat accentuated at the end of the experiments.

One month later GA and GAR were again tested, neither strain having been exposed to sulfanilamide in the interval. The findings were essentially the same as in Table I, the difference in susceptibility of the two strains to the drug being slightly more marked than before. An attempt was now made to modify the phase of GAR by growing it in enriched media. Twenty per cent ascitic fluid broth was used. The result was another variant, called GARA, which resembled the smooth type of organism in its colonial form except that unhemolyzed red blood cells were usually present immediately beneath the colony. Six weeks, and again three months, after the second test of GA and GAR all three strains were tested at the same time. The results of these and the original tests were represented in Chart 1.

At the close of these experiments GA was morphologically in the S or M-S phase, GARA was similar except for the unhemolyzed red blood cells beneath

the colony as noted before, and GAR was a definite S-R form very distinct in its colony type from either of the others. It is evident, therefore, that over a period of three months there was, in the case of GAR, a marked loss of resistance to sulfanilamide without any reversion to the standard or smooth type of this organism. It is also evident that in the case of GARA change of phase did not alter the susceptibility of the organism to sulfanilamide or cause any quicker loss of resistance to the drug than was the case with the parent strain GAR.

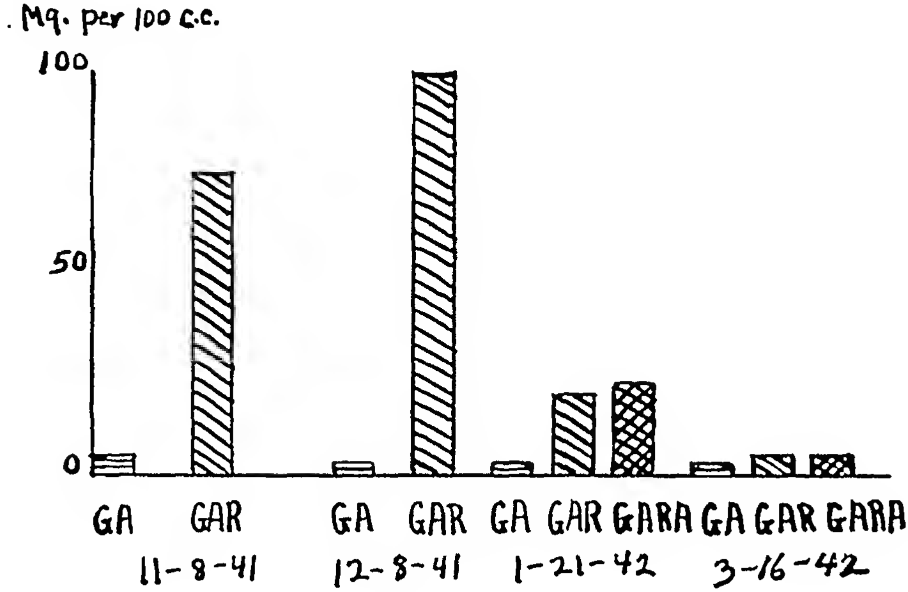


Chart I.

SUMMARY

1. A high degree of resistance to sulfanilamide was produced in vitro in a group A hemolytic streptococcus.

2. This resistance was maintained at a high level for one month and subsequently spontaneously disappeared in the course of about three more months.

3. Evidence is presented which suggests that though change of phase and drug resistance may be produced simultaneously by the action of sulfanilamide these two factors are not dependent upon each other, and may be separately modified.

REFERENCES

1. MacLeod, C. M., and Daddi, G.: A "Sulfapyridine-Fast" Strain of Pneumococcus Type I, Proc. Soc. Exper. Biol. & Med. 47: 69, 1939.
2. Lowell, F. C., Strauss, E., and Finland, M.: Observations on the Susceptibility of Pneumococci to Sulfapyridine, Sulfathiazole and Sulfamethylthiozole, Ann. Int. Med. 14: 1001, 1940.
3. Hadley, P., and Hadley, F. P.: Influence of Sulfanilamide on Mucoid and Smooth Phase Cultures of Hemolytic Streptococci in Vitro, J. Infect. Dis. 68: 246, 1941.
4. Dawson, M. H., Hobby, G. L., and Olmstead, M.: Variation in Hemolytic Streptococci, J. Infect. Dis. 62: 138, 1938.

CLINICAL EXPERIENCES WITH A MODIFICATION OF THE TAKATA REACTION IN BLOOD AND CEREBROSPINAL FLUID

H. Ucko, M.D., London, England

In 1935 and 1936 I described a modification of the Takata serum reaction. This reaction had been advocated as a test for the diagnosis of liver diseases by Staub (1929) and Jezler (1929). The modified test, which I studied in 160 cases, has since been investigated by a number of workers in different countries. They have tested the sera of more than 2,500 patients suffering from different morbid conditions, including a large number of pathologic conditions of the liver. In the Italian literature the test is generally referred to as "Ucko reaction." It has also been applied *to the cerebrospinal fluid of patients* suffering from organic diseases of the nervous system and has given interesting results in comparison with other protein reactions. It seems, therefore, worth while to survey the experiences of these authors, some of which are published in journals not available in this country and which give sufficient data to judge the diagnostic value of the test. This is the purpose of this paper.

TECHNIQUE AND NATURE OF THE MODIFIED TEST

The reaction consists in a precipitation of serum protein by addition of sodium carbonate and mercuric chloride to serum.

Owing to an omission in the first publication (1935), a misunderstanding has arisen about the number of tubes required for the test. This is how it should be carried out:

Reagents: 0.36 per cent solution of *anhydrous* sodium carbonate; 0.5 per cent solution of mercuric chloride (sublimate). Into 5 clean test tubes of 11 mm. diameter each, containing 0.2 c.c. of serum, are measured 0.1, 0.15, 0.2, 0.25, 0.3 c.c. of the sodium carbonate solution. After shaking, the same quantities of the mercuric chloride solution are added and the tubes are shaken again. Readings by daylight are taken at once and after ninety minutes:

Negative reaction: three or more tubes are *translucent. The mixture may be clear* or may show a slight opacity.

Reaction +: the first three tubes are turbid and *not translucent.*

Reaction + +: all tubes show a uniform turbidity and are *not translucent.*

Reaction + + +: a thick *precipitation* in all the tubes forms *immediately* after the addition of the reagents.

The performance of the test is extremely simple. Only the two solutions have to be prepared with absolute accuracy. If kept in the dark and at 0° C., they remain accurate for a month, after which time they have to be renewed. They should be checked from time to time by adding a drop of phenolphthalein to a mixture of equal parts of the solutions. If accurate, the mixture shows a weak pink color. It is important always to use *anhydrous* sodium carbonate.

Received for publication, April 20, 1942.

The nature of the reaction has not yet been cleared up satisfactorily. The active part of the reagent seems to be an unstable complex oxychloride of mercury, which precipitates serum protein under special quantitative, and perhaps qualitative, conditions (Ucko, 1936-1938).

The precipitate formed is serum globulin while albumin has a protective influence (Ucko, 1936). The protein concentration and the albumin-globulin ratio are, therefore, of definite importance. Other still unknown factors may play a part.

THE TAKATA REACTION

Takata's reaction, which is read after twenty-four hours, consists of a flocculation produced by addition of a strong solution of sodium carbonate, mercuric chloride, and fuchsin to a progression of serum dilutions and was designed for quite different purposes. It was Jezler who showed that this reaction is strongly positive in the sera of cirrhosis of the liver. Jezler, however, used Takata's procedure without adapting it to the new purpose. Extensive clinical studies have proved that positive Takata reactions are most frequently found in the sera of patients with liver diseases, especially with cirrhosis of the liver. Positive reactions may, however, occur in other illnesses. Multiple myelomas (Kahler's disease) always give a strongly positive reaction. Positive reactions can also be found in cases of glomerulonephritis, nephrosis, pneumonia, pulmonary tuberculosis, etc.

My procedure has the technical advantage of giving a clear end point only after ninety minutes and of allowing a gradation of the results from + to +++. A comparison of the two reactions in 132 cases by myself (1936) and of 33 cases by Guglielmini (1937) showed that the modified reaction possesses a greater certainty in indicating affections of the liver.

RESULTS OBTAINED WITH THE MODIFIED REACTION

Results are summarized in Tables I and II. The total number of cases is approximately 2,600, 373 of these being patients with liver or gall bladder lesions.

All but one of 92 cases of *Laënnec's atrophic cirrhosis* (Table I, *a*) had positive reactions (99 per cent). The reaction was +++ in 83 patients (90.2 per cent), ++ in 7, and + in one patient. Two of Cozzutti's cases had ++ reactions at an early stage. Statistics of the original Takata reaction (Schindel and Barth, 1934) have furnished only 83 per cent of positive and 6.8 per cent of doubtful (Jezler, 1938) results in liver cirrhosis.

Four cases of group *b* (Table I) had negative reactions: one with the diagnosis of chronic hepatitis (out of 4), one termed hypertrophic cirrhosis with splenomegaly (Takata's reaction negative), one hepatomegaly (Vaquez), and one hydatid cyst of the liver.

Fifteen of the 46 patients had +++ reactions. These consist of one patient with hypertrophic cirrhosis, one with amyloid degeneration of the liver, one with acute liver necrosis, 4 (of 16) with syphilitic hepatitis, one with arsenical hepatitis, one with liver abscess, one with multiple cysts of the liver, and 5 (of 7) with kala-azar. Regarding kala-azar, the formol test (gelatinization of the serum on addition of formol) may be remembered. It is due to a marked relative increase of the serum globulins (reduction of albumin?), a characteristic feature of kala-azar. Kala-azar has been included in this

group because the disturbed equilibrium of the serum proteins is probably due to the severe liver damage as a result of the protozoal infection.

One *reticulo-endothelioma* and 2 cases of *primary cancer* of the liver gave +, ++, and +++ reactions, respectively (Table I, *c*). The patient with a +++ reaction showed diffuse cirrhotic liver changes at autopsy.

TABLE I

NO.	DIAGNOSIS	−	+	++	+++	AUTHORS
a. 92	Cirrhosis of the liver (Laënnec)	1*	1*	7	83	Boccia, Cozzutti, Di Benedetto, Guglielmini, Jezler,* Sacchetti, Ucko
b. 1	Hypertrophic cirrhosis	–	–	–	1	Boccia and Gamalero
1	Hepatomegaly (Vaquez)	1	–	–	–	Sacchetti
1	Hypertrophic cirrhosis + splenomegaly	1	–	–	–	Guglielmini
1	Amyloid degeneration	–	–	–	1	Ucko
1	Acute liver necrosis	–	–	–	1	Boccia and Gamalero
1	Liver abscess	–	–	–	1	Boccia and Gamalero
1	Hydatid cyst of liver	1	–	–	–	Boccia and Gamalero
1	Mutiple cysts of liver	–	–	–	1	Boccia and Gamalero
16	Syphilitic hepatitis	–	6	6	4	Benedetto, Cozzutti, Sacchetti, Ucko
5	Enterogenous hepatitis	–	5		–	Benedetto
4	Chronic hepatitis	1	2	1	–	Cozzutti
1	Alcoholic hepatitis	–	1	–	–	Boccia and Gamalero
1	Arsenical hepatitis	–	–	–	1	Boccia and Gamalero
2	Amoebic hepatitis	–	–	2	–	Benedetto
2	Hepatitis brucellaris	–	–	2	–	Benedetto
7	Kala-azar	–	–	2	5	Benedetto
c. 2	Primary cancer of liver	–	–	1	1	Cozzutti, Ucko
1	Reticulo-endothelioma	–	1	–	–	Cozzutti
d. 31	Secondary cancer of liver	9	8	10	4(2)*	Benedetto, Boccia, Cozzutti, Guglielmini, Jezler,* Sacchetti, Ucko
1	Cancer of pancreas + jaundice	–	–	1	–	Cozzutti
1	Cancer of bile ducts	–	1	–	–	Boccia and Gamalero
e. 55	Liver congestion (heart failure)	7	29	18	1*	Benedetto, Cozzutti, Jezler,* Sacchetti, Ucko
1	Cardiohypertrophic cirrhosis	–	–	1	–	Boccia and Gamalero
f. 7	"Liver disorder?"	4	1	2	–	Ucko
35	Cholecystitis	23	12	–	–	Sacchetti
28	Gall bladder disease	24	4	–	–	Cozzutti, Ucko
1	Obstructive jaundice (stone)	–	1	–	–	Cozzutti
26	Gallstones + cholecystitis	–	12	12	2	Sacchetti
6	Cholangitis	3	2	1*	–	Boccia, Sacchetti, Jezler*
g. 40	Simple jaundice	34	5	1	–	Boccia, Cozzutti, Guglielmini, Ornstein, Sacchetti, Ucko

*These results deviate from findings of majority of authors. See text.

Among 33 cases of *secondary carcinoma* of the liver of different origin (Table I, *d*), there were 9 negative and 4 +++ reactions. Twenty patients had + and ++ reactions. This gives a total of 24 positive reactions (73 per cent), while in a similar group of 85 cases only 30 per cent of positive Takata reactions were observed (Schindel and Barth, 1934).

All authors agree that the degree of positivity of the reaction in patients with *liver congestion* due to heart failure corresponds with the clinical condition (Table I, *e*). Only 7 of 56 cases gave negative reactions (12.5 per cent). In comparison, 44 of 45 patients with a failing heart without clinical signs of liver congestion had negative reactions (Cozzutti, Guglielmini).

The next group (Table I, *f*) contains 103 patients, mainly with lesions of *gall bladder* and *bile ducts*. Fifty-four negative reactions were found. In 3 cases with gall bladder trouble and + reactions (Cozzutti) the liver was tender and enlarged and jaundice was present. Among the 4 cases labeled "liver disorder?" only the 3 patients with positive reactions showed signs of a lesion of the liver on examination.

Sacchetti's cases with 12 ++ and 2 +++ reactions will be discussed later.

The results obtained in cases of *simple* (catarrhal) *jaundice* (Table I, *g*) are important since most patients (34 of 40) had negative reactions. Very divergent results have been obtained with Takata's reaction in cases of jaundice. Six of my own cases had positive Takata and negative modified reactions. In some cases of jaundice positive results were the first signs of more severe liver damage (Ucko, 1936). No clinical details are available about the 6 positive cases in this group.

Di Benedetto, without quoting exact figures, states that most of his patients with simple jaundice had negative reactions, some, however, + and ++ reactions. His observations have not been included in Table I.

Table II

Number		Classification	–	+	++	+++	
1.	230	Liver diseases and diseases with definite liver damage (Table I, *a-e*)	21 (9.1%)	49	51	104	=45%
				$\underbrace{\quad}_{5}$ =90.9%			
2.	143	Diseases of the biliary system, uncertain liver cases, and simple jaundice (Table I, *f, g*)	88 (61.5%)	37	16	2	=1.4%
				=38.5%			
3.	2,031 (approx.)	Other diseases	1,752 (Approx. 86.3%)	128	111	40	=2%
				=13.7%			

Taken all together, 90.9 per cent of the cases with liver affections had positive reactions while only 38.5 per cent of positive results were obtained in patients suffering from diseases related to the biliary system and from simple jaundice. The number of +++ reactions in the first group was 104 compared with only 2 in the second group (45 per cent against 1.4 per cent). Also the percentage of ++ reactions is higher in group 1 than in group 2 (23 per cent against 11 per cent) (see Table II).

Patients suffering from all common internal and neurological illnesses, as well as a number of healthy people, have been examined by the different authors in order to find out how far positive reactions are related to affections of the liver. The total number of cases (2,031) is only approximate, since many authors do not quote the exact number of their patients.

Table III gives a list of nonhepatic diseases in which positive results have been obtained. They do not occur regularly. Many patients suffering from the same illnesses have negative reactions. The number of diseases is limited, and they are on the whole those in which the original Takata reaction has also been found to give positive results.

Of the patients examined 86.3 per cent had negative reactions, 11.7 per cent + and ++ reactions, and only 2 per cent +++ reactions. The majority of

patients with ++ and +++ reactions consisted of 104 cases of malaria observed by Vesce and of 25 skin diseases observed by Bronzini. The remaining cases are 17 with ++ and only 5 with +++ reactions; 3 patients with Kahler's disease (multiple myelomas), one with diabetes and one with miliary tuberculosis, all observed by Jezler and his collaborator, Bots. None of the other authors have found +++ reactions among their nonhepatic cases. The majority of these patients had + reactions (6.3 per cent).

TABLE III

NONHEPATIC DISEASES WITH POSITIVE REACTIONS

DEGREE OF REACTION +	++	+++
Nephritis, nephrosis	Nephritis, polyarthritis	Multiple myeloma (Jezler)
Diabetes	Rheumatoid arthritis	
Pernicious anemia	Diabetes	Diabetes (Jezler, Bots)
Peliosis rheumatica	Pernicious anemia	
Carcinosis, carcinoma of pancreas	Lymphogranuloma inguinalis	
Malignant lymphadenoma	Tuberculosis of lungs, miliary tuberculosis	Tuberculosis of lung (Rubino)
Tuberculosis of lungs, pleurisy	Lung abscess, bronchiectasis	Miliary tuberculosis (Jezler)
Pneumonia, lung abscess		
Typhoid, amoebiasis, brucellosis		
Malaria	Malaria, spinal tumor, tabes, general paralysis of the insane	Malaria (Vesce)
Cerebrospinal syphilis, hemiplegia	Tuberculosis of skin, other skin diseases	Skin diseases (Bronzini)
General paralysis of the insane (G.P.I.)		

DISCUSSION

The experiences of four authors could not be included in this general survey. An article by Montalbano (1938) on the subject was unobtainable. According to Vesce (1939), this author has examined 167 patients and has found positive results in most of his liver cases. He has, however, observed +++ reactions in patients without clinical signs of liver disease.

Backert's results (1938) have been omitted because he found only one negative reaction among 125 patients with different illnesses and 10 healthy persons. This fact can only be explained by an error in his technique. Backert himself is satisfied with his results, especially with regard to liver disease.

Ornstein (1937) has not recorded the degree of positivity of the reaction. Apart from the diseases in Table III, he has encountered positive results in cases with the diagnosis of pellagra, Grave's disease, and ovarian deficiency.

Rubino's (1938) readings of the reaction differ from those put forward by those mentioned and in previous papers. His results have, therefore, not been included in Table II and are dealt with separately.

Most authors come to the conclusion that the test is a very valuable, simple, and rapid laboratory method, generally indicating hepatic involvement and thus aiding clinical diagnosis.

Cozzutti emphasized that +++ reactions may occur in cases of syphilitic hepatitis and are not specific for liver cirrhosis. Table I shows other liver affections with +++ reactions.

Guglielmini regards the new reaction as being superior to the original one and believes that reaction +++ indicates cirrhotic changes; reaction ++, local-

ized liver affections; and reaction +, simple liver congestion. This classification is not confirmed by the result of the other authors.

Sacchetti followed up cases with chronic affections of the biliary system. His number of strongly positive reactions (++ and +++) is very high. According to him, the test is of great value in indicating possible damage of the liver parenchyma. Except in an emergency, patients with gallstones should not be operated on if their serum reaction is stronger than +. In such cases medical treatment should be given first. This may lead to an improvement of the test and to better chances if surgical interference is necessary. Two of Sacchetti's cases with +++ reactions died soon after operation. The postoperative recovery of cases with ++ reactions was unsatisfactory. After successful operations the reaction became negative.

Jezler, who introduced the Takata reaction into the clinic as a liver function test, and his assistant, Bots, are not in favor of modifications of the original technique. Bots' and Jezler's liver cases are identical and have, therefore, not been included separately in Table I. Jezler's results have been marked with an asterisk to show that they generally deviate from the findings of the majority of authors (– and + reactions in liver cirrhosis, +++ reaction in liver congestion, and ++ reaction in cholangitis). He also found +++ reactions in one patient with diabetes and one with miliary tuberculosis. With regard to the negative reaction in one patient with liver cirrhosis among 92 (Table I), it is interesting to note that a series of 25 Takata-negative cases, verified at autopsy, includes 2 cases of liver cirrhosis (Jezler, 1938, p. 431).

Although positive results with the test described are not confined to liver diseases, the great number of cases collected by at least 14 different authors show clearly the enormous incidence of positive reactions when the liver is primarily or secondarily involved in the morbid condition. Positive results in nonhepatic cases are comparatively rare. There is, however, no doubt that the reaction, like the original Takata reaction, can be positive in cases without clinical or pathologic manifestations of liver disease. The test is, therefore, not a specific indication of liver disease. It can only be used as such in connection with the clinical findings. It indicates an alteration of the serum proteins of a nature which is not properly understood, but it is possible to conclude that the liver must play an important part in the etiology of this alteration. If used with the necessary precaution and in connection with the clinical observation, and if certain other causes and diseases (Table III) have been excluded, the test is undoubtedly an easy method of great value for diagnosis, prognosis, and treatment of liver disorders, and for the differential diagnosis of doubtful cases. On the strength of the observations collected in this paper it seems justified to recommend the reaction for the routine examination of possible liver affections and as a test for the liver function with regard to protein metabolism. Reactions +++ suggest the presence of a severe parenchymatous lesion or destruction of the liver. In the majority of cases this lesion is cirrhotic in nature. Other severe liver lesions of an extensive but not always diffuse character can also give +++ reactions. Marked impairment of the protein-regulating function of the liver, recognizable by a positive serum reaction, may, however, occur without gross morphologic lesion. Even a strongly positive reaction is reversible and can be affected by treatment, as in the case

of syphilitic hepatitis and liver congestion. The test is also of assistance for the differential diagnosis of jaundice since the majority of cases of simple jaundice give negative reactions. Cases of jaundice with ++ or +++ reactions should be diagnosed with caution. All my own cases with such reactions turned out not to be simple jaundice. The differential diagnosis between simple jaundice and mild hepatic necrosis, often impossible for the clinician, seems also to be facilitated by the test (see also Ucko, 1938).

TABLE IV

NUMBER	DIAGNOSIS	RESULTS	EFFECT OF TREATMENT
21	Acute and generalized exudative eczema	+++ 10 cases	Cured
10	Eczema rubrum madidans	++ 9 cases	Improved
5	Papulous eczema	+ 2 cases	Not improved
39	Different chronic eczemas	+++ 3 cases	Improved
10	Hyperkeratotic eczema of palms and soles	++ 3 cases	Cured
15	Squamous eczema	+ 5 cases	Not improved
100		32	

The occurrence of positive reactions in nonhepatic diseases suggests that alterations of the serum proteins, similar to those found most regularly in liver diseases, can take place in certain other illnesses (Table III). Some of these (e.g., diabetes, pernicious anemia, typhoid, malaria, amoebiasis, brucellosis, syphilis) even affect the liver morphologically. The involvement of this organ may account for the disturbance of the protein metabolism manifesting itself in a positive serum test. Other diseases (e.g., nephritis; Kahler's disease; focal, acute, and chronic infections like rheumatoid and other forms of arthritis; pneumonia; pleurisy; lung abscess; bronchiectasis; tuberculosis; lymphogranuloma) show no such implication of the liver. A disturbed protein metabolism in Kahler's disease, in renal and certain infectious diseases is, however, known. It has not been recognized until recently that the liver plays an important role in the regulation of protein metabolism. Liver function tests (glucose, levulose, and galactose tolerance, hippuric acid formation, etc.) have shown impaired liver function in illnesses where no hepatic involvement was expected. There is strong evidence that the liver is responsible for the alteration of the serum proteins in hepatic diseases. While the matter is open to discussion, it is very likely that the liver is also responsible for the disturbance of the protein metabolism in those nonhepatic diseases where such a disturbance is a regular occurrence. The alteration of the serum proteins, which leads to a positive serum reaction, would then be the manifestation of a liver disturbance brought about by the causative agent. Apart from its practical significance the test, therefore, draws attention to the liver in diseases with disturbed protein metabolism and to the role of this organ in protein synthesis and equilibrium.*

SPECIAL INVESTIGATIONS

Some authors have used the test for determining the liver function in certain illnesses. Their results merit special consideration.

* [illegible] with the cephalin-cholesterol flocculation test (Hanger, F. W.: J. Cli[illegible] 1, 1939; Rosenberg, D. H.: Arch. Surg. 43: 231, 1941) give results almos[illegible] obtained by the modified Takata test, and it will be interesting to compare both reactions on the same cases. The similarity of the results of the two different tests in certain nonhepatic diseases points to a liver dysfunction rather than to an unspecific alteration in such cases.

1. *Malaria.* Vesee (1939) examined 137 cases of African malaria. The incidence of positive reactions was surprisingly high. He found 82 ++ and 22 +++ reactions. Provocation by injection of a concentrated liver extract and adrenalin and by violent physical exercise had no appreciable influence on the results. To explain the great number of positive results, Vesee puts forward the theory that a positive reaction indicates a functional disturbance of the reticulo-endothelial cells rather than of the liver cells, the former being permanently damaged by the malarial infection. This theory is interesting and could perhaps be further substantiated by experimental damage to the reticulo-endothelium without involving the liver cell, but the strongly positive reaction in cirrhosis of the liver with extensive destruction of the liver parenchyma and, on the other hand, a weakly positive reaction (+) found in a case of reticulo-endothelioma (Table I, *c*) does not render this conception very probable.

2. *General paralysis of the insane.* Angrisani (1937) examined the sera of 95 cases of general paralysis of the insane. Fifty-four patients had negative reactions, 3 had +, 2 had ++ reactions. No +++ reaction was found. The five patients with positive reactions all had clinical signs of liver damage, either of specific or postmalarial origin. Similar results were obtained by the author with van den Bergh's and with Bengal red test. He concludes that liver damage in general paralysis of the insane, occurs rarely and disputes the observations of Cassiano (1935), who found frequently positive results using the original Takata reaction.

3. *Pregnancy.* The majority of pregnant women examined by Zambonini (1938) had negative reactions. Among 120 cases of normal pregnancy only 11 (9 per cent) had a + reaction. No ++ or +++ reactions were found. Reactions + occurred most frequently during the third and sixth months of pregnancy. There was no difference between primiparas and multiparas. Unfortunately, no examinations were made in cases of pathologic pregnancy or toxemia.

4. *Skin diseases* (Eczema). Bronzini (1938) studied the reaction in 100 cases of different eczematous conditions. The number of strongly positive reactions is unusually high and throws doubts on his experimental technique. Patients with positive tests were also treated for possible liver damage, but the author does not state anything about the nature of this treatment. His results are included in Table II and are in detail in Table IV. If they can be confirmed by others, they would represent an important contribution to our conception of eczematous conditions.

5. *Lung tuberculosis.* Rubino (1938), who examined 200 cases of pulmonary tuberculosis, compared the results of the test in 36 patients with the amino acid curve in the blood after ingestion of glycine, a procedure which is used as a liver function test. The author does not give exact figures. He states that 30 per cent of his cases had negative reactions, 30 per cent had + and ++ reactions, and 40 per cent had +++ reactions. The latter include "immediate and delayed +++ reactions," but since only an immediate precipitation can be regarded as a +++ reaction, Rubino's classification of results may be ignored. Positive reactions in cases of lung tuberculosis have been found by other authors but never in such a high percentage of cases. Rubino states that the degree of positivity in his series corresponded with the severity of the tuberculous lesion. He, therefore, regards the test as a useful addition to other

methods for diagnosing the degree of activity of the tuberculous process. He noted that the result of this test and tests of the blood sedimentation rate were usually parallel. This fact has not been observed by other authors. He found, however, positive reactions with a normal sedimentation rate. The glycine reaction showed disturbed liver function in an unspecified number of cases, but the author's reaction corresponded with the glycine test in only 50 per cent of the cases. Rubino concludes that the reaction furnishes no indication of a liver deficiency: he disregards the fact that both tests measure different types of liver function. The importance of his observations lies in the close relationship he found between positive reactions and the severity of the tuberculous lesion and in the demonstration of a damaged liver function in cases of lung tuberculosis. Disturbances of protein metabolism in cases of severe tuberculosis have been demonstrated earlier; the albumin fraction of the serum is generally diminished; the globulin fraction, and especially fibrinogen, is increased. This may well be due to damage to the liver and impairment of its regulation of protein metabolism.

THE CEREBROSPINAL FLUID

Takata and Ara (1936) applied the original test to the cerebrospinal fluid and found it to be positive mainly in cases of syphilis of the nervous system. The Takata reaction has, however, not become a routine test for the examination of the cerebrospinal fluid because it had no advantages in comparison with other protein reactions and the results were not found to be specific for syphilis.

Three authors studied the new modification of the test on the cerebrospinal fluid. Zanetti's paper (1938) "Neurosyphilis and Ucko Reaction" was not obtainable in this country.

Ornstein (1937) examined the cerebrospinal fluid of 46 cases of general paralysis of the insane, and of 12 cases of tabes and compared the results with those of the Wassermann and Meinicke tests. The cerebrospinal fluid of 22 patients with negative Wassermann and Meinicke reactions were tested in comparison. His results are summarized as follows:

NUMBER	DIAGNOSIS	WASSERMANN		MEINICKE		UCKO	
		+	–	+	–	+	–
46	General paralysis of the insane	46	–	46	–	46	–
12	Tabes	8	4	8	4	8	4
22	Other affections	–	22	–	22	–	22

These data show a complete correspondence of the three reactions, and Ornstein concludes that the test is more valuable than Meinicke's and other reactions owing to its simple technique.

A very important contribution was made by Fleischhacker (1938). This author used only one tube, adding the lowest concentration, i.e., 0.1 c.c. of each reagent to 1.0 c.c. of cerebrospinal fluid. If the cerebrospinal fluid is free from blood and is not contaminated by bacteria, a positive reaction (marked opacity or precipitation) appears between forty-five and ninety minutes. Fleischhacker examined 153 cases of treated and nontreated general paralysis of the

insane and 24 different nervous and mental disorders and compared the results with the reactions of Weichbrodt, Pandy, Lange, and Wassermann.

In 61 cases of general paralysis of the insane all the reactions were positive. In 92 treated cases the results were as follows:

UCKO		LANGE AND PANDY		WASSERMANN		SERUM—WASSERMANN	
+	−	+	−	+	−	+	−
36	36	0	92	10	82	31	61

Twenty-four nonsyphilitic cases gave the following reactions:

DIAGNOSIS	UCKO		LANGE		PANDY	
	+	−	+	−	+	−
Cerebral tumor	2	−	−	2	−	2
Cerebral thrombosis	1	−	−	1	−	1
Disseminated sclerosis	1	−	−	1	−	1
Mental deficiency	3	3	−	6	−	6
Organic disorder?	7	4	−	11	2	9
Angioma cerebri	−	1	−	1	−	1
Posttraumatic psychosis	−	1	−	1	−	1
Fracture of skull	−	1	−	1	−	1

It can be concluded from the results that the reaction is not specific for syphilis but indicates generally abnormal protein conditions in the cerebrospinal fluid. Without being hypersensitive the test is more sensitive and, therefore, superior to Pandy's protein reaction usually made in the routine examination of the cerebrospinal fluid. The reaction may still be obtained—as in the case of treated general paralysis of the insane—when all other findings are apparently negative. It would be of interest to test cases of mental disorders, since Fleischhacker found positive reactions in cases of mental deficiency indicating lesions not detectable with the usual methods.

SUMMARY

The experience of different authors on approximately 2,500 cases with a simple and rapid modification of the Takata reaction is summarized and discussed.

Ninety per cent of patients suffering from liver diseases had positive reactions while at least 86 per cent of negative reactions were encountered in patients suffering from other illnesses. The test which indicates an alteration of the serum proteins seems to measure the liver function with regard to protein metabolism. Certain nonhepatic diseases frequently, but not always, give positive reactions. The problem of whether a positive test in these illnesses means a disturbance of the protein-regulating function of the liver or whether it occurs independently is discussed.

The experience of a number of authors with the test in cases of general paralysis of the insane, pregnancy, skin diseases, and tuberculosis is given in detail.

The reaction, which has also been applied to the cerebrospinal fluid, seems to be more sensitive than the routine tests for protein. It will, therefore, be of value in detecting minor alterations of the cerebrospinal fluid in disorders of the nervous system.

REFERENCES

Angrisani, D.: La reazione di Ucko nel paralitici progressivi, Rinasc. med. 14: 736, 1937.

Backert, P.: Die Takata-Reaktion nach Ucko in ihrer Bedeutung für den prakt. Arzt, Zentralbl. f. inn. Med. 59: 689, 1938.

di Benedetto, V.: La reazione di Ucko (modifica alla R. di Takata-Ara) nelle epatopatie, Boll. d. Soc. med.-chir., di Catania 5: 321, 1937.

Boccia, D., and Gamalero J.: La reaccion de Ucko en las enfermedades hepáticas, Prensa méd. argent. 25: 281, 1938.

Bots, P.: Über neue Modificationen der Takataschen Leberfunktionsprobe, Schweiz. med. Wchnschr. 68: 1230, 1938.

Bronzini, M.: La reazione di Ucko in dermatologia per la prova della funzionalità epatica, Riforma med. 54: 337, 1938.

Cassiano, P.: Applicazioni della reazione di Takata in psichiatria, Schizofrenie 4: 151, 1935.

Cozzutti, G.: Sul valore pratico di una nuova prova di funzionalità epatica (reazione di Ucko), Diag. e. tec. di lab. 7: 249, 1936.

Fleischhacker, H. H.: Takata Reaction on Cerebrospinal Fluid in Syphilis of Nervous System, J. Ment. Sc. 84: 378, 1938.

Guglielmini, G.: La reazione di Ucko nello studio della funzionalità epatica, Rinasc. med. 14: 548, 1937.

Jezler, A.: Die neuen Modificationen der Takataschen Leberfunktionsprobe, Verhandl. d. Gesellsch. f. Verdauungs. 14: 428, 1939.

Jezler, A.: Beitrag zur functionellen Leberdiagnostic, Ztschr. f. klin. Med. 111: 48, 1939.

Montalbano, S.: Sul valore clinico della reazione di Ucko, Folia med. 24: 134, 1938.

Ornstein, I.: Sur la valeur de la réaction de Takata-Ara modifiée par Ucko, dans le sérum sanguin et dans le liquide céphalorachidien, Compt. rend. Soc. de biol. 126: 519, 1937.

Rubino, A.: (Sassari) Sul valore della reazione di Ucko studiata in confronto con la curva amino-acidemica da carico, nei malati di tubercolosi polmonare, Policlinico (sez. prat.) 45: 841, 1938.

Sacchetti, A.: La reazione di Ucko nelle epatopatie, Diag. e. tec. di lab. 9: 705, 1938.

Schindel, L., and Barth, E.: Die Bedeutung der Takata-Reaktion für die Diagnose der Lebererkrankkungen in ihrem Verhältnis zur Galaktose-u. Bilirubin-Belastung, Klin. Wchnschr. 13: 1329, 1355, 1934.

Staub, H.: Über funktionelle Leberdiagnostik, Schweiz. med. Wchnschr. 59: 308, 1929.

Takata, M., and Ara: Liquorreaktion, Tokyo, 1926.

Ucko, H.: Zum Mechanismus der Reaktion nach Takata-Ara Bemerkungen zu der Arbeit von Schindel, Klin. Wchnschr. 14: 468, 1935.

—: Réaction de précipitation par le mercure dans le sérum des hépatiques, Compt. rend. Soc. de biol. 118: 534, 1935.

—: Serum Test for Diagnosis of Liver Disturbances, Guy's Hosp. Rep. 86: 166, 1936.

—: Untersuchungen zum Mechanismus der Takata-Reaktion (Leber-und Eiweissstoffwechsel), Klin. Wchnschr. 15: 1074, 1936.

—: Importance of Serum Proteins for Result of Takata Reaction, Acta med. Scandinav. 97: 557, 1938.

—: Catarrhal Jaundice, Brit. M. J., Nov. 5, p. 960, 1938.

Vesce, C.: La rezione di Ucko nella malaria cronica, Diag. e. tec. di lab. 10: 250, 1939.

Zambonini, H.: La funzionalità del fegato in gravidanza saggiata con la prova di Ucko, Riv. ital. di ginec. 21: 161, 1938.

Zanetti, G.: La reazione di Ucko applicata al liquor cefalorachidiano, Rassegna di studi psichiat. 27: 1033, 1938.

THE EFFECT OF ASCORBIC ACID (VITAMIN C) ON THE SENSITIVITY TO SALICYLATES IN A CASE OF RHEUMATIC FEVER

Louis Pelner, M.D., Brooklyn, N. Y.

A SEVERE case of rheumatic fever that rapidly became intolerant to salicylates was observed recently. The intolerance was manifested by a marked ringing in the ears that continued in spite of the reduction of the drug to a very low and ineffective amount. At about the same time, the occurrence of several severe nosebleeds, as well as a positive tourniquet test, and a low plasma ascorbic acid content, suggested the use of large amounts of ascorbic acid. The previous diet was analyzed and was found to be markedly deficient in vitamin C. Dramatically enough, the nosebleeds and the severe tinnitus ceased after forty-eight hours of vitamin C therapy and did not return, although the salicylates were increased to the initial high level and were continued in this manner for ten additional days.

Some observers[1] have pointed to a deficiency of vitamin C in acute rheumatic fever. This deficiency may predispose the person to toxic reactions to certain drugs, e.g., in this case salicylates. In any event, the toxic reaction ceased when vitamin C was given in adequate amounts.

CASE REPORT

P. V., a 15-year-old male, suddenly developed a high fever (104° F.); rapid pulse rate (130), pain in both shoulders, and dyspnea. The boy had no history of any previous serious disease and was in excellent health until the onset of the present illness. The mother is a rheumatic cardiac with both mitral and aortic murmurs, and a recent history of an attack of myocardial failure.

Examination of the patient revealed a rough, to-and-fro leather-like murmur over the base and apex of the cardiac area. There were no signs in the lungs or elsewhere. There was no swelling, redness, or limitation of motion in the shoulder joints, or in any other joints of the body. The pain in the shoulder joints was apparently due to a reflex mechanism. The to-and-fro pericardial friction rub was evanescent, lasting only a few hours. The sedimentation time was extremely rapid, twenty minutes for an 18 mm. fall. The blood count was white blood cells 15,200, polymorphonuclear leucocytes 85 per cent, lymphocytes 12 per cent, monocytes 3 per cent, hemoglobin 85 per cent, red blood cells 4,350,000. A diagnosis was made of rheumatic carditis.

Sodium salicylate was started at the rate of 15 grains (1 Gm.) every four hours combined with 5 grains (0.3 Gm.) of sodium bicarbonate. In addition, 60 grains (4 Gm.) of sodium salicylate was given by rectum once a day. On the third day of illness and salicylate medication, the patient developed a nosebleed and severe ringing of the ears. During the next two days the tinnitus continued severely in spite of the reduction of salicylate medication to 15 grains a day by mouth. The nose bled profusely during this time. A tourniquet test showed twelve petechiae in a 2.5 cm. circle after fifteen minutes of positive pressure. The plasma ascorbic acid content was 0.4 mg. per 100 c.c. (Farmer and Abt[2] method—normal value equals 0.7 mg. per 100 c.c.) It was, therefore, decided to give the patient large amounts of vitamin C. In addition, the diet of this patient over the preceding four weeks was reviewed and was found to be markedly lacking in foods containing vitamin C.

One hundred milligrams of ascorbic acid were given by mouth three times a day. Within forty-eight hours the nosebleed, as well as the ringing in the ears, had ceased. The salicylates were then stepped up to the former doses without development of tinnitus, or indeed

Received for publication, April 23, 1942.

any other sign of intolerance. The combined medication was continued for ten days without any ill effect. Urine examination was entirely negative for signs of renal irritation. The patient made an uneventful recovery, with a residual mitral systolic murmur as a sequel.

COMMENT

There is some evidence to show that in the guinea pig, chronic scurvy with a superimposed infection of β-streptococci produces lesions similar to those found in acute rheumatic fever.[1, 3] Scurvy alone does not produce the characteristic lesion. Clinically also, the ascorbic acid level of the blood plasma was found to be low in a group of patients[1] with rheumatic fever. It can easily be shown that rheumatic fever is a disease of the poor and undernourished,[4] and also that latent scurvy and rheumatic fever occur late in winter and early in spring, during the very same period that vitamin C is less widely available in foods.[5] However, the treatment of rheumatic fever patients with vitamin C in adequate amounts has proved disappointing.[6] Perla and Marmorston[7] believe that this does not rule out the connection between vitamin C and rheumatic fever, for vitamin C deficiency might enable the streptococcus to gain a foothold in these patients. Once this has been attained, vitamin C itself cannot affect the pathologic picture or prevent a recurrence. These authors feel that there exists some relationship between the lower vitamin C content of the blood and rheumatic fever, even though the connection has not been incontrovertibly proved as yet.

There also appears to be much evidence that an excess of vitamin C is a prophylactic against the toxic effects of neoarsphenamine and some other drugs. Vitamin C has been used with success in the control of reactions of certain patients to neoarsphenamine. In these persons, there may have been an insufficiency of the vitamin in the diet, and its value may be due to this fact. Recently, Marin[9] observed cases of mercurial and bismuth stomatitis among syphilitic patients, and administered ascorbic acid since it was decreased in the blood and urine. The ascorbic acid cured ten cases of this complication.

In this study the presence of a large amount of vitamin C in a patient who had a low serum ascorbic acid level, and other evidences of vitamin C deficiency appeared to remove the evidence of salicylate toxicity (marked tinnitus). At the same time marked nasal hemorrhages ceased wtihout recourse to packing or cauterization. It is suggested by one author that the hemorrhagic manifestations of rheumatic fever are due to a vitamin C deficiency.[1] In the study of bismuth and mercury sensitivity cited,[9] the author stated that vitamin C was necessary for the metabolism of these substances in the body; if it was not present in sufficient amounts toxicity would supervene. This may also be true in the case of salicylate sensitivity. This is suggested by the following simple experiment:

EXPERIMENT

To a test tube containing 5 drops of 1 per cent methylene blue solution in 5 c.c. of water a few crystals of ascorbic acid are added. On heating the tube the blue solution is immediately decolorized and a white solution results. The methylene blue is apparently reduced by the ascorbic acid. To another test tube containing 5 drops of 1 per cent methylene blue solution in 5 c.c. of

water, some sodium salicylate crystals are added, and the resulting mixture is allowed to stand. Even though vitamin C is later added in large amounts, as high as 50 to 100 mg., and the resulting solution is heated, no decolorization results. The vitamin C has apparently been fixed by the sodium salicylate and the methylene blue is not reduced.

It is possible that in the body sodium salicylate has an affinity for ascorbic acid and binds it, so that less and less is available for other functions. However, this conclusion cannot be drawn from the foregoing simple experiment.

Whatever the relationship of the lack of ascorbic acid to the toxicity of sodium salicylate, it would seem to be worth while to study the problem further, to see whether it is widely applicable. Certainly it can be said that in this case, saturation of this patient with large amounts of vitamin C (normal daily requirement is approximately 50 mg.)[10] enabled the continuation of salicylate medication and appeared to have a salutory affect on the nosebleed.

CONCLUSIONS

1. A severe case of rheumatic carditis became intolerant to sodium salicylate early in the course of the illness.
2. A low plasma ascorbic acid content, positive tourniquet test, and severe nosebleed suggested the presence of vitamin C deficiency.
3. The preceding diet of this patient proved to be deficient in vitamin C.
4. After the vitamin C intake was increased, sodium salicylate was again given in large amounts with impunity.

REFERENCES

1. Rinehart, J. F., Connor, C. L., and Mettier, S. R.: Further Observations on Pathological Similarities Between Experimental Scurvy Combined With Infection and Rheumatic Fever, J. Exper. Med. 59: 97, 1934.
   Rinehart, J. F., Greenberg, L. D., and Christie, A. U.: Reduced Ascorbic Acid Content of Blood Plasma in Rheumatic Fever, Proc. Soc. Exper. Biol. & Med. 35: 350, 1936.
   Rinehart, J. F.: Studies Relating Vitamin C Deficiency to Rheumatic Fever and Rheumatic Arthritis, Ann. Int. Med. 9: 671, 1935.
2. Farmer, C. J., and Abt, A. F.: Ascorbic Acid Content of Blood, Proc. Soc. Exper. Biol. & Med. 32: 1625, 1935.
3. Rinehart, J. F., and Mettier, S. R.: Heart Valves and Muscle in Experimental Scurvy With Superimposed Infection, With Notes on Similarity of Lesions to Those of Rheumatic Fever, Am. J. Path. 10: 61, 1934.
   Rinehart, J. F.: The Valves in Experimental Scurvy, and in Scurvy With Superimposed Infection, Am. J. Path. 9: 932, 1933.
4. Swift, H. F.: Factors Favoring the Onset and Continuation of Rheumatic Fever, Am. Heart J. 6: 625, 1931.
5. Gothlin, G. F.: A Method of Establishing the Vitamin C Standard and Requirements of Physically Healthy Individuals by Testing the Strength of Their Cutaneous Capillaries, Skandinav. Arch. f. Physiol. 61: 225, 1931.
6. Schultz, M. P.: Studies of Ascorbic Acid and Rheumatic Fever. II. Test of Prophylactic and Therapeutic Action of Ascorbic Acid, J. Clin. Investigation 15: 385, 1935.
7. Perla, D., and Marmorston, J.: Natural Resistance and Clinical Medicine, Boston, 1941, Little, Brown & Co., page 1057.
8. Sulzberger, M. B., and Oser, B. L.: Influence of Ascorbic Acid of Diet on Sensitization of Guinea Pigs to Neoarsphenamine, Proc. Soc. Exper. Biol. & Med. 32: 716, 1934.
   Cormia, F. E.: Experimental Arsphenamine Dermatitis, Influence of Vitamin C in Production of Arsphenamine Sensitiveness, Canad. M. A. J. 36: 392, 1937.
   Landfisch, S.: Synthetic Ascorbic Acid New Auxiliary Remedy in Arsphenamine Therapy, Polska gaz. lek. 16: 575, 1937.
9. Marin, J. V.: Treatment of Mercurial and Bismuth Stomatitis by Ascorbic Acid, Rev. méd. del Rosario 31: 1127, 1941.
10. Alexander, H.: Vitamin C and Tuberculosis, Deutsches Tuberk.-Bl. 14: 125, 1940.

177 KINGSTON AVENUE

EFFECT OF TRASENTIN A, TRASENTIN, AND MORPHINE ON RESPIRATION*

N. TOBOLOWSKY, M.D., DONALD SLAUGHTER, M.D., T. U. JOHNSON, M.D. AND R. E. VAN DUZEN, M.D., DALLAS, TEXAS

IT HAS been shown that trasentin is antagonistic to the actions of morphine on the bladder,[1] and recent experimental evidence has furnished confirmation of such action in regard to trasentin A on the isolated ureter.[2]

As it is possible that trasentin and its chemical allies are capable of inhibiting certain morphine effects other than on the bladder and ureter, it seemed of interest to investigate the relationship of effects on respiration using trasentin, trasentin A, and morphine in various combinations. Morphine and trasentin are both used therapeutically in certain combinations, such as ureteral colic.

PROCEDURE

In this work, 58 experiments were performed on dogs and 21 on cats. Respiration was recorded by means of a blunt intrapleural trocar connected to a sensitive tambour. The details of this simple, but very satisfactory, device are shown in Diagram 1. This apparatus may be used on unanesthetized as well as on anesthetized animals. When used in the former instance, a small amount of 1 per cent procaine hydrochloride is injected beneath the skin between the fifth and sixth interspace in the midaxillary line. A very small incision is then made through the skin with a scalpel, and the trocar is pushed with a craniad motion gently, but firmly, through the intercostal muscles and parietal pleura into the pleural cavity. It is very easy to accomplish this procedure without puncturing the lung, since the trocar falls into the space between the parietal and visceral pleura when resistance of the parietal pleura is overcome. Immediately upon entering the pleural cavity, respiratory excursions are recorded, provided no air has entered the cavity during the introduction of the trocar. To insure the prevention of pneumothorax, suction is applied to the T-tube after insertion and before withdrawal of the trocar. If air entered during the insertion of the apparatus and is removed by suction, it takes about three to four minutes for equilibrium to become established. We feel that this method is an excellent one to record direct respiratory movements, since there are no

*From the Department of Physiology and Pharmacology, Baylor University College of Medicine, Dallas.

Trasentin A is phenylcyclohexyl acetic acid ester and is available only for experimental use. It is now designated as trasentin 6-H.

Trasentin is diphenylacetic acid diethylaminoethanol ester.

Grant in aid to one of us (D.S.) given by Ciba Company, Summit, N. J., who also kindly furnished the trasentin A and trasentin used in this study.
Received for publication, April 24, 1942.

by-passes or hindrances and there is no lag in the recording of all respiratory waves. This method also has the advantage of being used many times for the same animal, thus making each animal his own control.

We at first tried without success to slow respiration with large doses of morphine given subcutaneously or intravenously in unanesthetized dogs. Finally, we made use of a standard technique of anesthetizing the dog with 26.1 mg. per kilogram of pentobarbital. As soon as the anesthesia was complete, the animal was placed on the table and the trocar was inserted and connected to the tambour. After suitable control respiratory records were made, 5 mg. per kilogram of morphine were injected intravenously. This method gave uniform slowing of respiration.

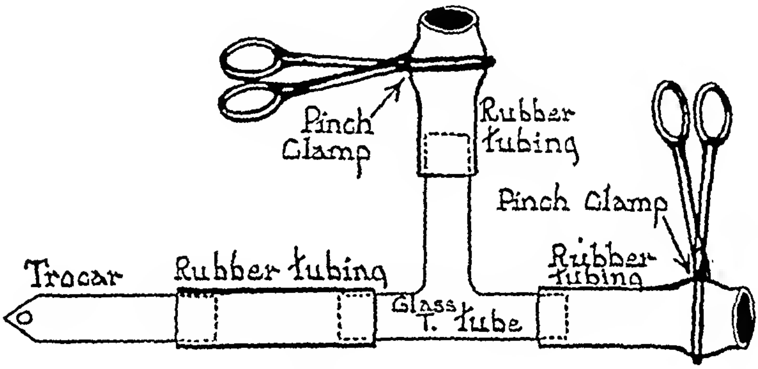


Diagram 1.—A schematic representation of the intrapleural trocar used in the experiments to record respiration.

In a certain number of acute experiments blood pressure and respiration were recorded simultaneously. Anesthesia was induced by pentobarbital, or barbital urethane administered intraperitoneally. All the cats and a few dogs used in this study were so treated.

As a rule, 6 mg. per kilogram of trasentin and 2 mg. per kilogram of trasentin A were given intravenously.

A few experiments were done on blood pressure alone, and the effects of trasentin A with and without intact vagi were compared.

RESULTS

In general, the results indicate that trasentin and trasentin A prevent depression of respiration by morphine when they are given before, after, or with morphine. When the trasentins are given with morphine, they never add to the depression of respiration produced by this opiate.

Fig. 1 illustrates well the effects of trasentin A and morphine on respiration of the dog. As noted on the tracings, these animals received pentobarbital previously.

Fig. 1A shows that 5 mg. per kilogram of morphine were given intravenously and (usually almost at once) marked depression of respiration ensued

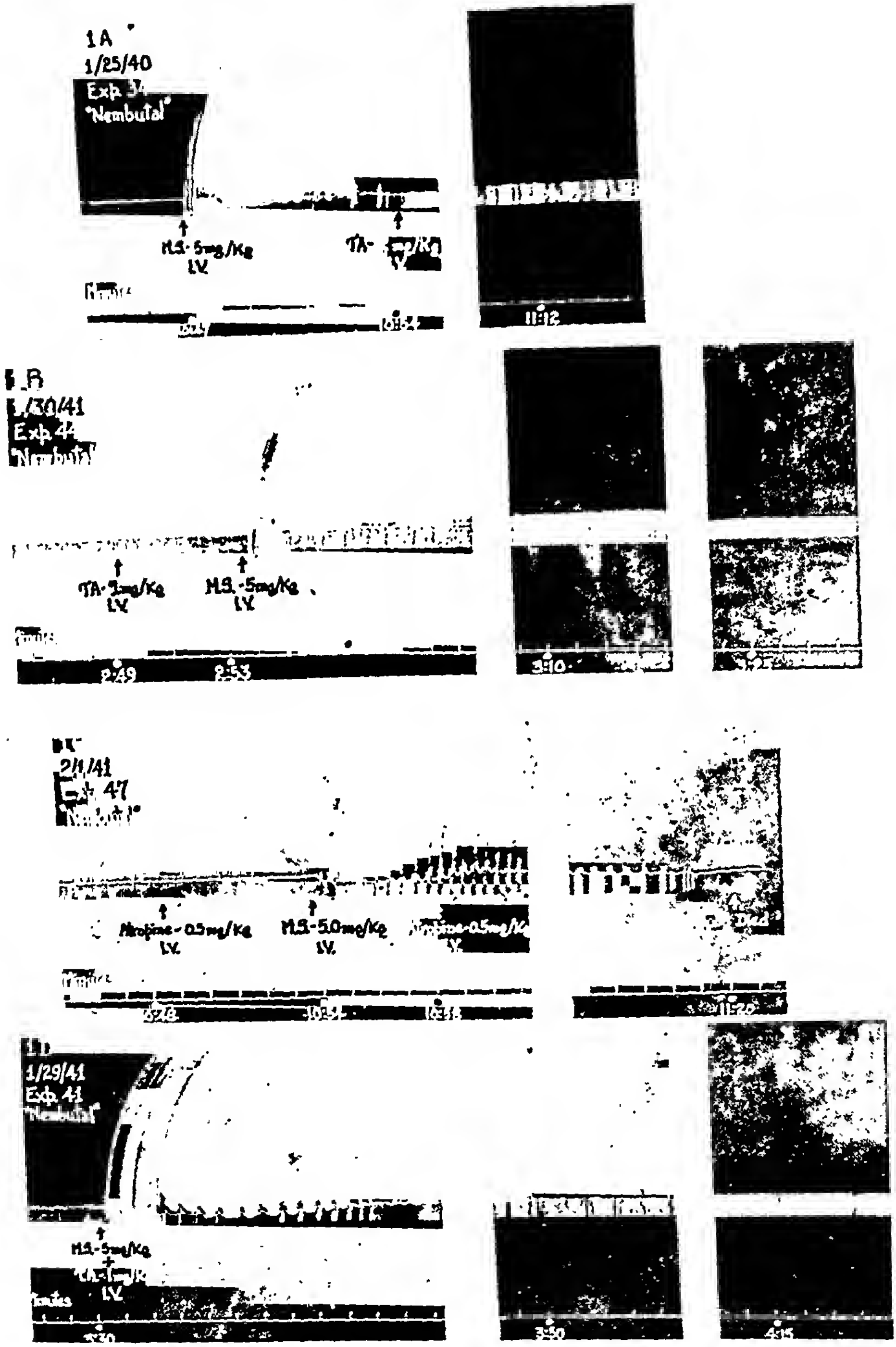


Fig. 1.—*A*, This tracing exhibits the effectiveness of trasentin A to counteract the depressant effect of morphine on respiration. Both drugs were given intravenously. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate. T.A. = trasentin A.

B, This tracing illustrates the ability of trasentin A to prevent the depressant effects on respiration by morphine. Both drugs were given intravenously.

C, This tracing illustrates the failure of atropine not only to prevent but also failure to correct depression of respiration by morphine. Both drugs were given intravenously. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate.

D, This tracing presents evidence that when morphine and trasentin A are given together the depressant effects of morphine on respiration are reduced. This was a smaller dose of trasentin A than that usually employed. Both drugs were given intravenously.

Note that in tracings shown in Fig. 1*A*-*D* there was at first a brief but sharp increase in respiration when morphine was administered.

following the brief stimulation. (This latter effect will be commented on in the discussion.) When 2 mg. per kilogram of trasentin A by intravenous injection were then given eight minutes later, the respiration returned to an amplitude and rate greater than the normal rhythm preceding the injection of morphine.

Fig. 1*B* shows that 2 mg. per kilogram of trasentin A administered intravenously four minutes before 5 mg. per kilogram of morphine entirely prevented depression of respiration by morphine. This is a very important finding since a large percentage of animals died after receiving such a dose of morphine alone. When one compares this preventative and corrective action of trasentin A with that of atropine, the difference is very striking. Fig. 1*C* demonstrates not only the failure of atropine in a dose of 0.5 mg. per kilogram intravenously to prevent the depressant effect of morphine on respiration, but also its failure to correct the depressant effect of this hypnotic as well. As a consequence, the animal died from the effects of the morphine. This was the usual finding in these experiments.

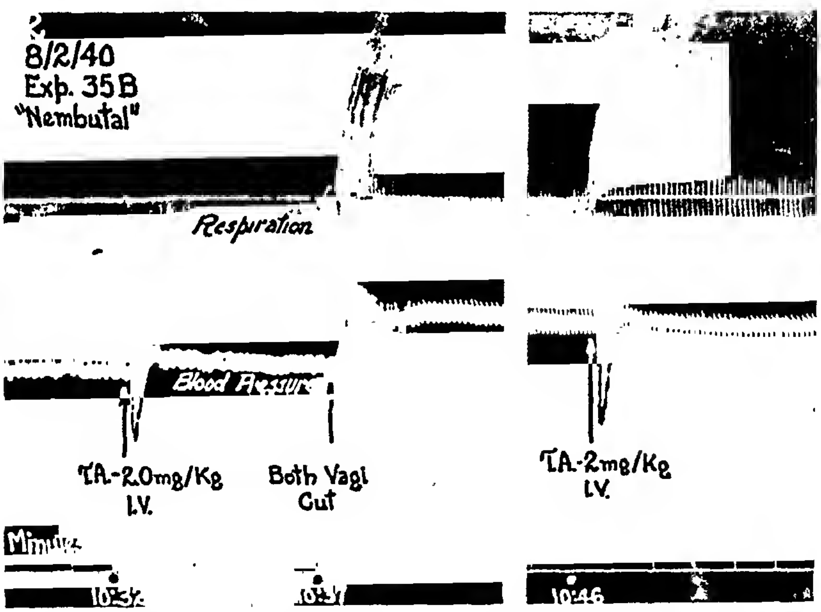
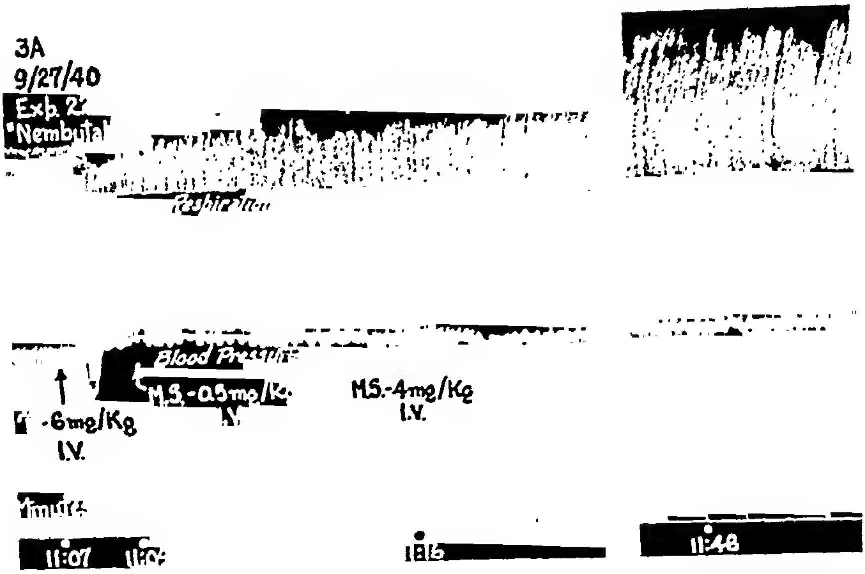


Fig. 2.—This tracing illustrates the effect of trasentin A on respiration and blood pressure. When given intravenously by itself, note the increased amplitude of respiration. The fall in blood pressure resembles that of acetylcholine and is present whether the vagi are present or divided. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate. T.A. = Trasentin A.

Fig. 1*D* shows a combination of 5 mg. per kilogram of morphine plus only 1 mg. per kilogram of trasentin A administered intravenously. Even with this small dose of the latter drug, the depressant effect of morphine on the respiration lasted only about nine minutes, and thereafter the respiration returned to above the normal rhythm when compared with the rate and amplitude before these drugs were given. This clearly demonstrates the protective action of trasentin A.

In other instances large subcutaneous doses of morphine alone were given and marked depression of respiration followed. Here, as already noted, trasentin A corrected the inhibitory effect of the hypnotic.



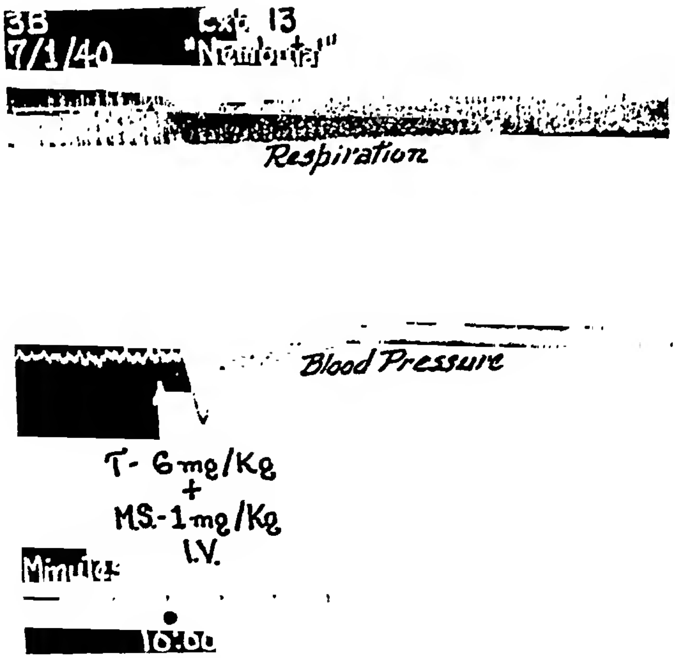


Fig. 3.—*A*, This tracing illustrates the effect of trasentin in preventing depression of respiration by morphine. Note the acetylcholine-like fall of blood pressure produced by trasentin. Both drugs were given intravenously. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate. T. = trasentin.

B, This tracing presents the constant finding of stimulation of respiration when trasentin and morphine are administered intravenously as a single dose. Note that the blood pressure did not fall to any greater extent than when trasentin was given by itself, as seen in Fig. 3*A*.

Results similar to these were obtained in the cat, but the effectiveness of trasentin A was not as marked, since in our hands morphine alone tended to stimulate respiration. However, it has been shown that large, repeated doses of morphine will produce respiratory depression in the cat and evidence of the ability of trasentin A to prevent this depression has been obtained.

Simultaneous records of blood pressure and respiration were made under pentobarbital anesthesia (Fig. 2). In this tracing 2 mg. per kilogram of trasentin A given intravenously produced an acetylcholine-like fall in blood pressure. The vagi were then divided, and the trasentin A administration was repeated. The fall in blood pressure was still present and of the same magnitude, and it is seen that the amplitude of respiration is improved. Other experiments using doses of atropine which would paralyze the vagi before the administration of trasentin gave similar results. These results indicate that trasentin A acts beyond the vagus.

Effects with trasentin and morphine were of a slightly different nature than with trasentin A and morphine, although they pointed in the same direction. Fig. 3*A* shows the effect of 6 mg. per kilogram of intravenous trasentin on a dog that was anesthetized with pentobarbital. The blood pressure change is similar to that of trasentin A, but the fall is not as great. This is of interest, since the dose of trasentin used was three times that of trasentin A. As noted in this tracing, the respiration was stimulated and successive doses of 0.5 and 4 mg. per kilogram of morphine given intravenously failed to produce any depression. This result, however, was not as consistent as that noted with trasentin A. Fig. 3*B* illustrates the most significant and constant effect of trasentin. Here intravenous trasentin and morphine given together always produced stimulation of respiration. In this instance, only 1 mg. per kilogram of morphine plus 6 mg. per kilogram of trasentin was used, but the latter dose in combination with larger doses of morphine (up to 6 mg. per kilogram) gave a similar effect. It will be noted that the combination of morphine and trasentin, as seen in Fig. 3*B*, gives a fall in blood pressure of no greater depth than the trasentin alone, as noted in Fig. 3*A*.

DISCUSSION

These results clearly indicate that trasentin A and trasentin when administered before, after, or with morphine do not add to any depression of respiration caused by morphine. This correlates well with the previously described effects of the trasentin on the activity of the bladder[1] and the ureter.[2] Hence we feel that *experimental* evidence in animals does not indicate that it is dangerous to give these drugs to human beings with or following morphine.

Maloney and Tatum[3] have reported that morphine increases the effectiveness of inhibitory vagal impulses on respiration in the rabbit. This suggests that morphine might depress respiration at least partly by a central, cholinergic action on the vagus. The possibility that morphine possesses central cholinergic actions has been postulated.[4] This work concerned potentiation of morphine relief of pain by prostigmine. Since this potentiation occurred and since morphine acts centrally, it was suggested that morphine had a central cholinergic effect. That there is a good possibility that central cholinergic transmission occurs has been suggested by Adam and others.[5] Since the trasentins are believed to be anticholinergic in activity as well as antimyonergic on smooth muscle, they may counteract morphine depression of respiration in this fashion. It is, of course, possible that they may also stimulate the respiratory center.

When one compares the effectiveness of the trasentins on respiration depressed by morphine, trasentin A (on basis of dosage) is three times as efficient.

Simultaneous records of blood pressure and respiration were made under pentobarbital anesthesia (Fig. 2). In this tracing 2 mg. per kilogram of trasentin A given intravenously produced an acetylcholine-like fall in blood pressure. The vagi were then divided, and the trasentin A administration was repeated. The fall in blood pressure was still present and of the same magnitude, and it is seen that the amplitude of respiration is improved. Other experiments using doses of atropine which would paralyze the vagi before the administration of trasentin gave similar results. These results indicate that trasentin A acts beyond the vagus.

Effects with trasentin and morphine were of a slightly different nature than with trasentin A and morphine, although they pointed in the same direction. Fig. 3*A* shows the effect of 6 mg. per kilogram of intravenous trasentin on a dog that was anesthetized with pentobarbital. The blood pressure change is similar to that of trasentin A, but the fall is not as great. This is of interest, since the dose of trasentin used was three times that of trasentin A. As noted in this tracing, the respiration was stimulated and successive doses of 0.5 and 4 mg. per kilogram of morphine given intravenously failed to produce any depression. This result, however, was not as consistent as that noted with trasentin A. Fig. 3*B* illustrates the most significant and constant effect of trasentin. Here intravenous trasentin and morphine given together always produced stimulation of respiration. In this instance, only 1 mg. per kilogram of morphine plus 6 mg. per kilogram of trasentin was used, but the latter dose in combination with larger doses of morphine (up to 6 mg. per kilogram) gave a similar effect. It will be noted that the combination of morphine and trasentin, as seen in Fig. 3*B*, gives a fall in blood pressure of no greater depth than the trasentin alone, as noted in Fig. 3*A*.

DISCUSSION

These results clearly indicate that trasentin A and trasentin when administered before, after, or with morphine do not add to any depression of respiration caused by morphine. This correlates well with the previously described effects of the trasentin on the activity of the bladder[1] and the ureter.[2] Hence we feel that *experimental* evidence in animals does not indicate that it is dangerous to give these drugs to human beings with or following morphine.

Maloney and Tatum[3] have reported that morphine increases the effectiveness of inhibitory vagal impulses on respiration in the rabbit. This suggests that morphine might depress respiration at least partly by a central, cholinergic action on the vagus. The possibility that morphine possesses central cholinergic actions has been postulated.[4] This work concerned potentiation of morphine relief of pain by prostigmine. Since this potentiation occurred and since morphine acts centrally, it was suggested that morphine had a central cholinergic effect. That there is a good possibility that central cholinergic transmission occurs has been suggested by Adam and others.[5] Since the trasentins are believed to be anticholinergic in activity as well as antimyonergic on smooth muscle, they may counteract morphine depression of respiration in this fashion. It is, of course, possible that they may also stimulate the respiratory center.

When one compares the effectiveness of the trasentins on respiration depressed by morphine, trasentin A (on basis of dosage) is three times as efficient.

days and 0.4 c.c. for the second period of thirty days; the dogs, 1 c.c. throughout the duration of the treatment. The methylglucamine was prepared as a 6.2 per cent solution, so that each rabbit received 12.4 mg. and each dog 62 mg. of this salt per dose; the glucophylline was prepared as a 10 per cent solution (in the formula the ratio of methylglucamine to theophylline is 1:16 to 1:18), so that each rabbit received approximately 10 mg. for thirty days and 20 mg. later, and each dog received approximately 50 mg. of each of the two constituents in the compound. On the basis of the initial weight the milligram per kilogram dose was then computed for each animal. This figure appears in the respective tables. Injections were all made intraperitoneally once daily. All the animals in the two species were kept under similar environmental conditions and were provided at all times with an excess of food and water. Weights were recorded at weekly intervals. With the exception of one control rabbit, which died on the twenty-fourth day of the experimental period, all animals lived to the end and without exception appeared to maintain normal health throughout. The dogs in the glucophylline group (No. 3) were definitely more

TABLE I

WEIGHT CHANGES IN DOGS

GROUP	ANIMAL	DOSE TOTAL	INITIAL WEIGHT (KG.)	54 DAYS FINAL WEIGHT (KG.)	% DECREASE	WEIGHT LOSS (KG.)
Control (Saline)	1	1 c.c.	8.3	7.7	7	0.6
	2	1 c.c.	11.4	10.9	4	0.5
	3	1 c.c.	11.1	10.1	9	1.0
Methylglucamine hydrochloride	4	6 mg./kg.	10.3	10.2	1	0.1
	5	6.7 mg./kg.	9.2	8.0	13	1.2
	6	5.4 mg./kg.	11.5	9.9	14	1.6
Glucophylline	7	10.8 mg./kg.	9.2	6.93	25	2.3
	8	10.1 mg./kg.	9.9	9.6	3	0.3
	9	10.3 mg./kg.	9.8	9.1	7	0.7

active and lively than the others. It is of interest to note that the animal caretaker, who had no knowledge of what the animals being injected were receiving, volunteered the observation that the group of rabbits which we were treating with glucophylline ate more food and drank more water than those making up the other group; and that the amount of feces and urine voided by these animals was considerably greater. Reference to Table II reveals a fairly uniform average percentage of weight gains for the several groups both from the initial doses and after the doses were doubled. In the first series the order of percentage increases for the thirty-day period was: no medication, 47; physiologic saline, 59; methylglucamine, 49.5; glucophylline, 49. By the end of the first thirty days, then, the animals in all groups had made substantial weight gains, some of them better than 50 per cent. This, no doubt, accounts for the gradual tapering off of weight increases during the second phase of the experiment when the doses were doubled. Most of the animals had already reached maturity, after which weight increase was due primarily to deposition of fat and only secondarily to actual body growth. The total group average percentage gains were: no medication, 136; saline, 150; methylglucamine, 139; and glucophylline, 128.

3. Maloney, A. H., and Tatum, A. L.: Study of Effect of Morphine Upon Respiratory Center, J. Pharmacol. & Exper. Therap. 40: 291, 1930.
4. Slaughter, Donald, and Gross, E. G.: Some New Aspects of Morphine Action; Effect on Intestine and Blood Pressure, Toxicity Studies, J. Pharmacol. & Exper. Therap. 68: 96, 1940.
5. Adam, H. M., McKail, R. A., Obrador, S., and Wilson, W. C.: Acetylcholine in Cerebrospinal Fluid, J. Physiol. 93: 45, 1938.
6. Barlow, O. W., and Gledhill, J. D.: Tranquilizing and Respiratory Depressant Effects of Tribromethanol (Avertin), Amylene Hydrate, Isoamylethylbarbituric Acid (Amytal), and Ethyl (1 Methyl-Butyl) Barbiturate (Pentobarbital) Alone and in Combination With Morphine on the Rat, J. Pharmacol. & Exper. Therap. 49: 36, 1933.
7. Plant, O. H., and Pierce, I. H.: Studies of Chronic Morphine Poisoning in Dogs, General Symptoms and Behavior During Addiction and Withdrawal, J. Pharmacol. & Exper. Therap. 33: 329, 1928.
8. Graham, J. P. D., and Lazarus, S.: The Action of the Synthetic Antispasmodics, "Trasentin" and Trasentin 6-H, J. Pharmacol. & Exper. Therap. 69: 331, 1940.
9. Meier, R., and Hoffmann, K.: Spasmolytische wirkung von basischen estern und amiden substituierter essigsauren, Helvet. med. acta, Supp. 6, 7: 106, 1941.

FURTHER STUDIES ON GLUCOPHYLLINE*

A. H. Maloney, Ph.D., M.D., Washington, D. C.

IN A PREVIOUS report on the diuretic activity of glucophylline by Maloney, Burton, and Robinson,[1] in which experimental data were presented regarding the action of this double salt of theophylline and methylglucamine, a xanthine compound exhibiting the very desirable properties of high diuretic efficiency with long duration of action, it was indicated that our study was being continued with the object of determining "whether glucophylline exerts any untoward effects on the liver or kidney." The present report sets forth these and other pertinent results obtained from this investigation. The study was conducted in three parts, viz., the effect on (1) weight, (2) blood, and (3) vital organs. Full-grown dogs and immature rabbits were employed.

1. Weight. A. Dogs: Nine full-grown dogs, ranging in weight from 8.2 to 11.5 kg., were divided into groups of three and treated with physiologic saline (controls), methylglucamine, and glucophylline, respectively. All the animals lost weight during the course of the fifty-four days of the experimental period. The percentage loss in each group was 6.7, 9.3, and 11.6 in the order named. We do not think the medicaments played any significant role. The determinants here were probably the new diet and the unaccustomed artificial environment of the kennels (see Table I).

B. Rabbits: Thirty-six female rabbits, ranging in weight from 0.8 to 1.7 kg., were employed. These were divided into three groups of twelve. Half of the animals in group 1 received no treatment whatever, while the other half received normal physiologic saline. These were used as two sets of controls. Those in groups 2 and 3 received methylglucamine and glucophylline, respectively. For convenience, all animals received equal volumes of the substances injected: the rabbits received 0.2 c.c. for the first period of thirty

*From the Department of Pharmacology, Howard University School of Medicine, Washington, D. C.

Received for publication, April 28, 1942.

days and 0.4 c.c. for the second period of thirty days; the dogs, 1 c.c. throughout the duration of the treatment. The methylglucamine was prepared as a 6.2 per cent solution, so that each rabbit received 12.4 mg. and each dog 62 mg. of this salt per dose; the glucophylline was prepared as a 10 per cent solution (in the formula the ratio of methylglucamine to theophylline is 1:16 to 1:18), so that each rabbit received approximately 10 mg. for thirty days and 20 mg. later, and each dog received approximately 50 mg. of each of the two constituents in the compound. On the basis of the initial weight the milligram per kilogram dose was then computed for each animal. This figure appears in the respective tables. Injections were all made intraperitoneally once daily. All the animals in the two species were kept under similar environmental conditions and were provided at all times with an excess of food and water. Weights were recorded at weekly intervals. With the exception of one control rabbit, which died on the twenty-fourth day of the experimental period, all animals lived to the end and without exception appeared to maintain normal health throughout. The dogs in the glucophylline group (No. 3) were definitely more

TABLE I

WEIGHT CHANGES IN DOGS

GROUP	ANIMAL	DOSE TOTAL	INITIAL WEIGHT (KG.)	54 DAYS FINAL WEIGHT (KG.)	% DECREASE	WEIGHT LOSS (KG.)
Control (Saline)	1	1 c.c.	8.3	7.7	7	0.6
	2	1 c.c.	11.4	10.9	4	0.5
	3	1 c.c.	11.1	10.1	9	1.0
Methylglucamine hydrochloride	4	6 mg./kg.	10.3	10.2	1	0.1
	5	6.7 mg./kg.	9.2	8.0	13	1.2
	6	5.4 mg./kg.	11.5	9.9	14	1.6
Glucophylline	7	10.8 mg./kg.	9.2	6.93	25	2.3
	8	10.1 mg./kg.	9.9	9.6	3	0.3
	9	10.3 mg./kg.	9.8	9.1	7	0.7

active and lively than the others. It is of interest to note that the animal caretaker, who had no knowledge of what the animals being injected were receiving, volunteered the observation that the group of rabbits which we were treating with glucophylline ate more food and drank more water than those making up the other group; and that the amount of feces and urine voided by these animals was considerably greater. Reference to Table II reveals a fairly uniform average percentage of weight gains for the several groups both from the initial doses and after the doses were doubled. In the first series the order of percentage increases for the thirty-day period was: no medication, 47; physiologic saline, 59; methylglucamine, 49.5; glucophylline, 49. By the end of the first thirty days, then, the animals in all groups had made substantial weight gains, some of them better than 50 per cent. This, no doubt, accounts for the gradual tapering off of weight increases during the second phase of the experiment when the doses were doubled. Most of the animals had already reached maturity, after which weight increase was due primarily to deposition of fat and only secondarily to actual body growth. The total group average percentage gains were: no medication, 136; saline, 150; methylglucamine, 139; and glucophylline, 128.

TABLE II

WEIGHT CHANGES IN RABBITS

GROUP	ANIMAL	DOSE TOTAL	INITIAL WEIGHT (KG.)	WEIGHT IN 30 DAYS (KG.)	% INCREASE	DOSE TOTAL	WEIGHT ON FINAL DAY (KG.)	TOTAL INCREASE WEIGHT (KG.)	TOTAL % INCREASE
Control (a) No medication	1	0	0.88	Died					
	2	0	0.93	1.81	95	0	2.86	1.94	179
	3	0	0.98	1.74	78	0	2.61	1.64	168
	4	0	0.98	1.60	65	0	2.02	1.04	107
	5	0	1.05	1.66	57	0	2.59	1.54	146
	6	0	1.23	1.08	−12	0	2.20	0.97	80
(b) Physiologic saline	1	0.2 c.c.	1.23	1.82	49	0.4 c.c.	2.60	1.38	112
	2	0.2 c.c.	1.18	1.84	55	0.4 c.c.	2.55	1.38	116
	3	0.2 c.c.	1.15	1.76	53	0.4 c.c.	2.57	1.42	123
	4	0.2 c.c.	1.05	1.75	66	0.4 c.c.	2.60	1.55	147
	5	0.2 c.c.	0.95	1.58	66	0.4 c.c.	2.82	1.87	197
	6	0.2 c.c.	0.80	1.32	65	0.4 c.c.	2.48	1.68	209
Methylglucamine hydrochloride		mg./kg.				mg./kg.			
	1	8.3	1.50	2.08	39	11.6	3.22	1.72	115
	2	8.4	1.48	2.27	54	10.8	2.50	1.02	70
	3	9.0	1.38	2.02	47	12.3	2.60	1.22	89
	4	10.6	1.18	1.35	15	17.7	2.70	1.53	130
	5	10.8	1.15	1.79	55	13.9	2.74	1.59	140
	6	11.0	1.13	1.16	2	21.6	2.36	1.24	110
	7	11.5	1.08	1.33	23	19.1	2.33	1.26	110
	8	11.8	1.05	1.60	52	15.5	2.43	1.38	130
	9	12.1	1.03	1.50	46	16.5	1.92	0.89	81
	10	12.7	0.98	1.56	60	15.5	3.16	2.19	225
	11	13.1	0.95	1.54	62	16.4	2.57	1.62	170
	12	15.5	0.80	1.82	137	13.8	3.26	2.46	300
Glucophylline	1	11.5	1.75	2.24	21	17.9	3.56	1.81	103
	2	12.5	1.60	2.34	45	17.1	3.25	1.65	103
	3	14.3	1.40	2.05	45	19.5	2.82	1.62	100
	4	14.3	1.40	2.12	50	18.9	3.24	1.84	132
	5	16.1	1.24	1.95	57	20.5	2.95	1.71	137
	6	16.7	1.20	1.96	63	20.4	2.67	1.47	121
	7	16.7	1.20	1.82	50	21.9	2.76	1.56	130
	8	16.7	1.20	1.62	35	24.9	2.65	1.45	120
	9	17.4	1.15	1.62	42	24.9	2.49	1.34	116
	10	18.2	1.10	1.58	44	25.0	2.79	1.69	153
	11	18.2	1.10	1.78	62	22.2	2.93	1.83	166
	12	20.0	1.00	1.73	73	23.1	2.52	1.52	150

TABLE III

COMPOSITE BLOOD VALUES OF DOGS AND RABBITS

ANIMAL VALUES	R.B.C. MILLIONS	W.B.C. THOUSANDS	DIFFERENTIAL PERCENTAGE					HB. %	N.P.N. %	CREATININE %	NUMBER OF SAMPLINGS
			NEUT.	LYMPH.	MONO.	EOS.	BASOPH.				
Dog: "Normal values"*	7.2	11.8	69	20	6	5	0.7	77	30.8	1.5	?
Saline	5.5	6.7	71	25	4	4	0	111	32.4	1.4	21
M-glucamine	5.3	8.5	70	26	6	0	0	109	26.1	1.3	21
Glucophylline	5.4	10.0	77	21	2	0	0	103	28.9	1.5	20
Rabbit: "Normal values"*	5.6	7.9	43	42	9	2	4.0	95	31.0	1.1	
No medication	6.3	7.5	17	66	3	13	1	100	30.7	1.6	11
Saline	5.9	6.7	15	60	4	21	0	102	46.9	2.0	12
M-glucamine	5.5	8.1	10	68.5	5	16	0.5	104	38.1	2.0	20
Glucophylline	5.0	7.4	22	63.0	4	11	0	99	39.5	2.0	20

*"Normal values" of blood (count and chemistry) reported above are taken from Tables I and III, respectively, from Wagoner, G., and Custer, R. P.: *A Handbook of Experimental Pathology*, Springfield, Ill., 1932, Charles C Thomas.

2. Blood: Studies on blood changes included (1) erythrocytes, (2) white blood cells, (3) differential, (4) nonprotein nitrogen, and (5) creatinine. One complete weekly determination was made on each dog during the fifty-four days of the experimental period. In the case of the rabbits there was one complete weekly determination on each of eight animals, two within each group, the last being made at the time of disposition of the animal for histologic purposes. As with the dogs, the figures appearing in Table III represent the composite averages within each group. Included also in this table, for purposes of comparison, are figures of normal blood values for dogs and rabbits.*

3. Histologic: Tissues fixed in 10 per cent formalin were prepared for microscopic study from both sets of animals. The dogs were all disposed of on their last experimental day (the fifty-fourth); the rabbits were disposed in series of twos at ten-day intervals, beginning with the end of the first experimental period on the thirtieth day. Thus, whereas the first group that were treated had received a single daily dose for thirty days, the second, third and fourth groups received this, followed afterward by a double dose daily at intervals of ten, twenty, and thirty days, respectively. Although these animals were exposed to unequal quantities of the drugs over varying periods of time, no appreciable differences could be demonstrated in the tissues studied. Pulmonary hemorrhages, demonstrable in most samples studied, were due to ante-mortem traumatic injury. Samples of brain, lung, heart, liver, kidney, and spleen were examined (see Table IV).

From these studies it is apparent that glucophylline neither disturbs the growth nor checks the normal increase in the weight of immature rabbits. Furthermore, with respect to both dogs and rabbits no noticeable difference could be observed in the general appearance of treated and untreated animals regarding health and general state of nutrition. In these respects all the groups exhibited the same general condition of health. The microscopic study revealed the presence of cloudy swelling as a rather constant finding in samples taken from both dogs and rabbits. This finding was quite as conspicuous in the tissues of animals used as controls, as it was in those of animals treated with methylglucamine and glucophylline. The blood samples taken from our rabbits showed an inordinately high differential eosinophilic count. This phenomenon is a matter of not uncommon occurrence in the blood of laboratory animals. It is very probable that these cells, or many of them, were pseudo-eosinophiles or emphophiles. Referring to this type of cell. Wagoner and Custer[2] make the observation that "the analogue of the human neutrophile varies only in that the granules are larger than neutrophilic granules and take the acid stain by preference, although there is a slight affinity for the basic dye." Since this eosinophilic condition was uniformly present in each group, treated as well as untreated, we felt that we were dealing with cases of pseudo-eosinophilia and that these findings, therefore, were probably of no pathologic significance. Indeed, many a laboratory animal that appears to be normal and healthy in every respect has been found to exhibit visceral lesions exposed by the microscope, if not grossly visible at autopsy. In the course of these studies one of

*Taken from figures compiled by Wagoner and Custer and reproduced in Tables I and III, pages 33 and 34 of their *Handbook of Experimental Pathology*, Springfield, Ill., 1932, Charles C Thomas.

TABLE IV

TISSUE CHANGES IN DOGS AND RABBITS

SPEC. ANIMAL	BRAIN	LUNG	HEART	LIVER	KIDNEY	SPLEEN
			A. Physiologic Salt Solution			
10. Dog 2	No change	No change	Slight cloudy swelling	Slight cloudy swelling	Slight cloudy swelling	Slight pigmentation. Slight increased cellularity in pulp
15. Rabbit A-4	No change	Hyperemia, small focal hemorrhages	Focal areas of necrosis with considerable inflammatory reaction	Cloudy swelling	Degeneration of tubular epithelium, especially in convoluted tubules	Slight hyperplasia
17. Rabbit A-3	No change	Slight hemorrhages	Cloudy swelling	Slight cloudy swelling	Hyperemia; cloudy swelling	Marked hyperemia
			B. Methylglucamine			
7. Dog 4	No change	No change	Cloudy swelling	Marked cloudy swelling	Cloudy swelling	Hyperplasia
8. Dog 5	No change	Diffuse hemorrhages throughout	No changes	Cloudy swelling	Cloudy swelling, marked in some tubules; necrosis in some tubules	Hyperplasia
11. Rabbit B-1	--	Diffuse hemorrhages into alveoli, bronchi, and interstitial tissue	Marked hyperemia; cloudy swelling	Cloudy swelling	Hyperemia; cloudy swelling	
19. Rabbit B-8	No change	Small hemorrhages	Cloudy swelling	Cloudy swelling; increase in periportal connective tissue	Marked hyperemia; cloudy swelling	Hyperemia
13. Rabbit B-12	Slight thickening of meninges. Focal hemorrhage—focal area of softening with cellular reaction	Small hemorrhages; arteriosclerosis	Multiple hemorrhages; cloudy swelling	Cloudy swelling	Cloudy swelling. Leucocytic infiltration in interstitial tissue of cortex. Hyperemia	Hyperemia
5. Rabbit B-3	No change	Hyperemia; arteriosclerosis	Small hemorrhages. Slight increase in perivascular connective tissue	Cloudy swelling; passive hyperemia; maybe some fat	Cloudy swelling	Slight hyperplasia

TABLE IV—CONT'D

SPEC. ANIMAL	BRAIN	LUNG	HEART	LIVER	KIDNEY	SPLEEN
			C. Glucophylline			
9. Dog 9	No change	Hemorrhages in focal areas, cells with vacuolated cytoplasm in interstitial tissue. Suggest fat, slight fibrosis	Cloudy swelling	Cloudy swelling	Cloudy swelling	No change
12. Rabbit C-7	Hyperemia; infiltration of small and large monocytes in meninges	Small granuloma	Slight cloudy swelling	Cloudy swelling	Cloudy swelling; hyperemia; arteriosclerosis of small arteries	Slight hyperplasia
14. Rabbit C-2	No change	Diffuse hemorrhages	Cloudy swelling	Cloudy swelling	No change	Hyperplasia
18. Rabbit C-5	No change	Hyperemia, few small hemorrhages	Cloudy swelling; small hemorrhages	Cloudy swelling	Marked cloudy swelling; hyaline casts in collecting tubules	Slight hyperplasia

the female dogs in our kennels gave birth to a litter of six pups. On the day after birth we began giving daily injections of glucophylline to three of these pups, using the other three as controls. No significant differences have been noticed, and at the end of thirty days the relative individual weights have shown uniform increases.

SUMMARY

Laboratory studies on dogs and rabbits treated with methylglucamine and glucophylline revealed:

1. Growth of immature rabbits is not disturbed. These animals made increases in size and weight commensurate with normal controls.
2. The dogs, treated and controls, all lost weight—a condition due probably to food and housing restraint.
3. Histologic study of brain, heart, lung, spleen, kidney, and liver of both dogs and rabbits showed no harmful effects that might be attributed to the drug.
4. Significant figures setting forth results are appropriately tabulated.

We wish to express thanks to Dr. Robert S. Jason and Dr. Bernard Kapiloff for personal assistance, and, to the Abbott Laboratories for providing the drugs used in these experiments.

REFERENCES

1. Maloney, A. H., Burton, A. F., and Robinson, J. W. L.: The Diuretic Action of Glucophylline, J. LAB. & CLIN. MED. 22: 600, 1937.
2. Wagoner, G., and Custer, R. P.: Handbook of Experimental Pathology, Springfield, Ill., 1932, Charles C Thomas.

CARCINOMA OF STOMACH DEVELOPING IN PERNICIOUS ANEMIA*

LEWIS H. BRONSTEIN, M.D., NEW YORK, N. Y.

THE combination of pernicious anemia and carcinoma of the stomach in one patient tends to rouse our curiosity as to the possible relationship between the two diseases. This inquisitiveness becomes more marked when one treats a patient for pernicious anemia and years later finds that that person has a carcinoma of the stomach. Many questions and possibilities arise. The following case is being reported as one that raises the discussion again. It is that of a patient who was treated for pernicious anemia for four and one-half years, at the end of which time an extensive carcinoma of the stomach was found. Table I shows the results of the various blood studies that were done during his course of treatments.

T. K., a 45-year-old Greek male, an unemployed kitchen helper, was admitted to the surgical service on Jan. 19, 1936, with a history of a mass in the right scrotum for some time. This suddenly became large and painful.

Examination revealed a tender scrotal mass, 7 by 6 by 5 inches, which transilluminated light and was moderately tender. There was discoloration of the distal third, the color being different from the remainder of the scrotum, suggesting to the surgeon that something recent had happened to it. Although the patient appeared pale, with a questionable lemon-yellow tint to the skin, immediate operation was decided upon. This was done under local anesthesia and a hydrocele with old and recent hemorrhages was found. The surgical condition cleared uneventfully.

The medical service then took over. The blood studies revealed a hyperchromic macrocytic anemia, as can be seen from the figures in the first line in Table I. The platelet count was normal. The red blood cell fragility test showed a slight increase in resistance, the figures ranging from 0.46 per cent sodium chloride to 0.28 per cent. The icteric index was 15. The sternal bone marrow was studied by Dr. Nathan Rosenthal, hematologist at the Mt. Sinai Hospital, who stated that the case was one of pernicious anemia. The Wassermann reaction was negative.

The gastric contents had no free hydrochloric acid on two occasions, histamine being used as a stimulant both times. They were of a heavy and mucoid character, with a total acid ranging from 7 to 12. No lactic acid was found. The benzidine test for blood was slightly positive on one occasion, but this was felt to be due to the trauma of passing the large stomach tube. Traces of urobilinogen were found in the urine. Stool examination did not show any occult blood, ova, or parasites.

The patient was treated with parenteral liver extract and a reticulocyte response of 12 per cent was obtained on the fifth to sixth day. It had varied from 0 to 1 per cent before the onset of treatment. A steady rise in the hemoglobin and red blood cell count took place. On discharge from the hospital on Feb. 26, 1936, the red cell count was 3,690,000 and the hemoglobin was 58.8 per cent. The cell volume index had dropped from 1.34 to 0.92. The icteric index had also dropped to 5.5 units.

*From the Medical Service, Beekman Hospital, New York City, Dr. Arthur H. Terry, Jr., Director.

Received for publication, May 9, 1942.

During his numerous visits to the clinic difficulties were encountered in trying to get cooperation, but these were mainly due to language differences. He was given biweekly, weekly, and monthly injections for the next four years, depending on the judgment of the physician who saw him in the medical clinic.

TABLE 1

DATE	NUMBER OF RED BLOOD COR-PUSCLES	PERCENT-AGE HEMO-GLOBIN	COLOR INDEX	MEAN CORPUSCULAR VOLUME	MEAN COR-PUSCULAR HEMOGLOBIN	MEAN COR-PUSCULAR HEMOGLOBIN CONCEN-TRATION
1/14/36	0.94	28	1.45	143	53.2	37.0
7/17/36	3.05	66	1.08	120	36.7	30.4
11/12/37	4.88	104	1.06	110.6	36.2	32.9
2/14/38	4.18	94	1.12	110	37.8	34.3
6/24/38	3.98	88	1.10	110.8	37.6	33.7
9/30/38	4.92	97	0.98	109.7	33.5	30.5
1/14/39	4.38	85	0.97	120	33	26
4/17/39	4.9	85	0.96	83	29	35
1/19/40	4.4	68	0.77	84	26	31

A neurologic examination was ordered as a routine procedure on Jan. 12, 1937, and the following findings were recorded: There was a loss of the pupillary light reflex. The biceps and triceps reflexes were diminished. There was a questionable diminution in vibratory sense along the shin bones and toes. A diagnosis of spinal cord disease, probably due to pernicious anemia, was made. However, a spinal fluid Wassermann was requested in order to rule out tabes dorsalis. This was done on Feb. 17, 1937, and the fluid showed a negative Wassermann and a normal colloidal gold curve. On Oct. 11, 1938, one and one-half years later, another neurologic examination was performed to determine the progress of the case, since liver extract had been given in the interim. This showed the pupils to react slightly to light. The biceps and triceps reflexes were still diminished. General sensory examination was negative. The conclusion was that there was probably no spinal cord disease.

The patient never had any complaints during his visits to the out-patient department. He suffered from the usual vicissitudes of existence in a slum, such as difficulty in obtaining extra food allowances. He was bitten by a dog in October, 1937, for which he was treated in the surgical out-patient department. In 1938 and 1939 he had all his upper teeth extracted and had to wait long for an upper dental plate. The plate proved to be a poor restoration and numerous repairs had to be made. During this period he had difficulty in eating a full diet and he lost some weight. His weight dropped from an average of 127½ pounds to 122½. However, in late 1939 and early 1940 his weight returned to its original figure.

In a two-week period in June, 1940, his weight dropped to 112 pounds, the lowest it had ever been. He was, therefore, watched closely, and by July his weight had decreased to 109 pounds. At this time some vague epigastric complaints were elicited by the physician in the medical clinic. In the gastrointestinal clinic he told the physician that he had had some epigastric pain for about two months. An x-ray of the stomach was ordered, and it indicated a large new growth in the middle portion of the stomach, on its greater curvature side (see Fig. 1).

At first he refused admission to the hospital but he later consented to enter. The weight at that time was 107½ pounds. Physical examination showed only emaciation and pallor. The blood showed hypochromic anemia, as seen by a red blood cell count of 2,880,000 and a hemoglobin of 40 per cent. A gastric analysis showed the free hydrochloric acid to be absent. Lactic acid was present. A four-plus reaction for blood was obtained. The stools were consistently positive for occult blood.

Exploratory laparotomy was advised in the hope that an operable lesion would be found. The patient refused this and left the hospital. The social service department kept an eye on him since he refused to return to the out-patient department for check-ups. He was admitted to Bellevue Hospital on Dec. 2, and died on Dec. 11, 1940.

AUTOPSY PROTOCOL

The autopsy protocol was furnished through the kindness and cooperation of Dr. Douglas Symmers, Director of Pathology, Bellevue Hospital:

The examination of the gastrointestinal tract reveals the lower one-third of the esophagus to be infiltrated with tumor tissue. The lumen is not constricted. The tumor tissue invades the mucosa and the underlying tissue of the esophageal wall. This tumor mass extends by direct continuity with the tumor growth seen in the stomach. There is no distention of the esophagus above the tumor site. The stomach is constricted in its midportion, resembling an "hourglass." On opening the stomach the lumen is found to contain a large amount of clotted blood. There is seen an ulcerating and fungating tumor mass which extends from the greater curvature over the entire posterior surface and around to the lesser margin of the stomach, leaving the anterior surface of the stomach free of tumor tissue.

Microscopic examination of the stomach tissue showed one part of the section to be replaced completely by tumor tissue. This tumor tissue extends from the mucosa, invades the underlying submucosa, muscularis, and serosa. Only small remnants of the aforementioned tissue are present. The tumor cells assume, for the most part, no set arrangement. Some, however, tend to assume a papillary form, and others, an acinar formation. The cells have no definite shape. They are fairly large cells with scant amount of eosinophilic cytoplasm and a large central oval vesicular nucleus with large nucleoli. Many mitotic figures are seen. Some of the tumor tissue has undergone necrosis.

The tail of the pancreas was found to be invaded for a short distance by the stomach tumor. Metastatic nodules were found in the liver, left adrenal, and surrounding lymph nodes. Microscopic examination showed this tissue to be similar to that in the stomach. No tumor cells are noted in the bone marrow. The different blood cells are present in their normal numbers.

The final diagnosis was as follows: Carcinoma of the gastrointestinal tract; fungating and ulcerating carcinoma of the stomach with hourglass constriction and hemorrhage into the lumen; extension into liver, pancreas, and esophagus; and metastases to mesenteric lymph nodes, liver, and left adrenal; moderate coronary atherosclerosis; mild atherosclerosis of the aorta; focal adhesive pleuritis; mild congestion of the liver; and general emaciation.

DISCUSSION

This case raised several interesting questions. One was whether or not this patient had had carcinoma of the stomach at the original discovery of his hyperchromic macrocytic anemia. The anemia was discovered accidentally when he was admitted to the hospital for a hemorrhage into a hydrocele. He had no complaints referable to his gastrointestinal tract. The only disconcerting fact was that he did not have an atrophic tongue. However, the various factors in the blood examination, such as color index, mean corpuscular volume, mean corpuscular hemoglobin, and bone marrow smears seemed to point to a diagnosis of pernicious anemia. The achlorhydria was a confirming factor, as were also the icteric index of 15 units and the response to liver treatment. The absence of occult blood in the stool at his first examination made us believe that there was no point in doing an x-ray of the gastrointestinal tract merely as a routine. This point, of course, cannot be settled in this patient. The type of carcinoma that was found, namely, a fungating, adenocarcinoma in the fundus of the stomach in the greater curvature side, has been called the "silent type" because it does not produce symptoms till very late in the course of the disease. It is still possible, therefore, that it might have been present all the time.

Some patients have been reported in the literature in whom gastric complaints have appeared during the course of pernicious anemia and in whom gastrointestinal x-rays were reported as showing no pathology. Subsequently, these patients developed carcinomas in various portions of the stomach. Conner and Birkeland[1] reported three such patients in whom carcinomas were found, respectively, in the esophagus and cardia of the stomach, in the pylorus, and in an unstated site in the third patient. Miller[2] reported two patients in whom carcinomas were found in the pylorus and in the middle portion of the greater

Fig. 1.

and lesser curvatures of the stomach. Of the seven patients reported by Washburn and Rozendaal[3] in whom a clinical diagnosis of pernicious anemia antedated the discovery of the carcinoma of the stomach, four patients had had previous x-rays of the stomach which showed no pathology. One patient had a scirrhous carcinoma of the upper two-thirds of the stomach; the second, a polypoid, ulcerating lesion removed by subtotal gastrectomy; the third, carcinoma of the distal half of the stomach; and the fourth, polypoid carcinoma involving the greater portion of the stomach.

Although no analysis has been made of the patients who had routine x-rays of the stomach, even though no gastrointestinal symptoms were present, it might seem advisable to perform such an examination on any patient who has the symptom complex, pernicious anemia. It, of course, might be advocated to examine any patient with pernicious anemia with a gastroscope, since lesions can be observed earlier with it than with an x-ray examination. However, the value of this procedure has not been demonstrated as yet in this type of case.

Of great interest in the case presented here is the change in the blood picture, which occurred some time before either loss of weight or gastric symptoms could be elicited. The figures in April, 1939, showed a marked drop as compared to the previous calculations. In January, 1940, this was even more marked with a hemoglobin of 68 per cent. At that time a search should have been made for some complications. Some authors[4, 5] have from time to time pointed out the necessity of searching for malignancies when the type of anemia in a treated case of pernicious anemia changes to a normocytic or microcytic form. Whether an operable carcinoma would have been found is a moot point. It is also impossible to state whether any lesion would have been discovered, although the size of the lesion found in July, 1940, makes me feel that some pathology would have been seen in January, 1940.

The literature records very few cases in which the blood count showed the suspicious changes before symptoms appeared. In the greatest majority of reported cases it was the appearance of new symptoms, or the inability to alleviate these complaints with liver therapy, which led to the x-ray studies of the gastrointestinal tract. One of the patients reported by Conner and Birkeland (Case 9)[1] had pernicious anemia for seven years when fresh blood was noted in a gastric analysis. Two x-rays taken two months apart were negative. A blood count done three months after the last x-ray examination, and following an attack of influenza, showed a hemoglobin of 29 per cent and an erythrocyte count of 2 million. Three months later he vomited blood, and an x-ray examination showed an obstructive lesion in the esophagus. Here the blood count changed to a low color index anemia before the x-rays disclosed a definite lesion. Numerous reports[1, 3-6] are present in the literature where a low color index anemia was discovered at the time that the carcinoma was found in the patients with pernicious anemia.

An interesting point and one which can lead to a great deal of speculation is to be found in the bone marrow studies. A bone marrow puncture, performed at the beginning of the observation, showed the cells to be typical of pernicious anemia. Nevertheless, study of the bone marrow at autopsy revealed no apparent changes.

CONCLUSIONS

A case of pernicious anemia is reported in which a carcinoma of the stomach was found four and one-half years after the initial diagnosis was made. Of particular interest is the fact that blood studies showed a change in the type of anemia several months before signs or symptoms of the carcinoma were found. It is suggested that blood studies be done repeatedly on patients with pernicious anemia and that a prompt search for pathology be made should a change in the character of the anemia be found.

REFERENCES

1. Conner, H. M., and Birkeland, I. W.: Coexistence of Pernicious Anemia and Disease of the Gastro-Intestinal Tract, Ann. Int. Med. 7: 89, 1933.
2. Miller, T. G.: Addisonian Anemia and Carcinoma of the Stomach in the Same Individual, Internat. Clin. 1: 167, 1935.
3. Washburn, R. N., and Rozendaal, H. M.: Gastric Diseases Associated With Pernicious Anemia, Ann. Int. Med. 11: 2172, 1938.
4. Held, I. W., and Goldbloom, A. A.: Carcinoma of the Stomach and a Cured Case of Addison-Biermer's (Pernicious) Anemia, J. A. M. A. 108: 1398, 1937.
5. Liechti, H.: Zweite Krankheit bei Pernicioeser Anaemie, Schweiz. med. Wchnschr. 69: 172, 1939.
6. Cabot Case 144442: Development of Gastric Symptoms in a Patient Totally Relieved of Pernicious Anemia by Liver Therapy, New England J. Med. 199: 1274, 1928.

CLINICAL CHEMISTRY

GUANIDINE AND ITS RELATIONSHIP TO MUSCULAR DYSTROPHY*

Robert P. MacFate, Ch.E., M.S., Ph.D.
Chicago, Ill.

THE abnormal metabolism of creatine (methylguanidine acetic acid) in progressive muscular dystrophy and certain other muscle conditions, as reported by Levene and Kristeller,[1] Brand and his co-workers,[2] Magee,[3] Thomas, Milhorat, and Techner,[4] Milhorat,[5] Tripoli and Beard,[6] and others, led me to study the effects of the administration of simple guanidine salts on creatine metabolism. It was first thought that administered guanidine might give rise to an added production and excretion of creatine. As the experiments progressed, the results suggested that the increased excretion of creatine following guanidine administration and in nutritional muscular dystrophy was due to changes produced in the muscle rather than to a direct production of creatine from the added guanidine.

MINIMUM LETHAL DOSE OF GUANIDINE HYDROCHLORIDE AND METHYLGUANIDINE SULFATE

Guanidine hydrochloride and methylguanidine sulfate were chosen for study. The minimum lethal doses of these compounds were determined to serve as a basis for their administration. The minimum lethal dose (M.L.D.) was defined as the number of milligrams of guanidine salt per gram of body weight which would cause the death within twenty-four hours of one or more of a series of fasting animals.

Guanidine hydrochloride and methylguanidine sulfate were administered intramuscularly and by stomach tube in a 5 per cent aqueous solution to healthy guinea pigs that had fasted twenty-four hours. The findings at the chosen end points are shown in Table I. Guinea pigs receiving the M.L.D. of either guanidine salt became nervous, showed varying degrees of trembling and twitching, and finally went into convulsions and died. Guinea pigs receiving less than the M.L.D. showed varying degrees of nervousness, trembling, and twitching, depending on the amount of guanidine salt given. Death occurred in some of the guinea pigs in this latter group, but later than twenty-four hours after the administration of the guanidine.

*From the Laboratory of Physiological Chemistry, University of Illinois College of Medicine, Chicago.

This paper is based on a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

Received for publication, June 3, 1942.

PROTECTIVE ACTION OF GLYCINE

Schmeideberg and Bunge[7] were among the first to show that benzoic acid is detoxicated in the kidney by conjugation with glycine and then excreted as the less toxic hippuric acid. Since that time it has been found that this synthesis takes place in other parts of the body as well as the kidney. It also has been found that the animal organism protects itself in the same manner from many toxic substances, such as phenylacetic acid, cresols, and others. Perhaps this same mechanism comes into play in the detoxication of simple guanidine salts.

Single and daily doses of 1 mg. of glycine per gram of body weight were found to be nontoxic in healthy guinea pigs. The protective action of this amount of glycine was then studied.

TABLE I

FATALITY OF THE M.L.D. OF GUANIDINE SALTS

GUINEA PIGS USED	SALT ADMINISTERED	METHOD OF ADMINISTRATION	M.L.D. (MG. PER GM. OF BODY WEIGHT)	ANIMALS DIED IN 24 HOURS (PER CENT)
14	Guanidine hydrochloride	Intramuscular	0.24	60
13	Guanidine hydrochloride	Oral	0.40	80
10	Methylguanidine sulfate	Intramuscular	0.29	75
10	Methylguanidine sulfate	Oral	0.50	50

TABLE II

PROTECTIVE ACTION OF SINGLE DOSES OF GLYCINE AND GLUCOSE AGAINST GUANIDINE HYDROCHLORIDE

TOTAL NUMBER OF GUINEA PIGS	GUANIDINE HYDROCHLORIDE	GLYCINE	GLUCOSE	NUMBER OF GUINEA PIGS SURVIVING	PER CENT SURVIVING	AVERAGE BLOOD GLUCOSE IN SURVIVORS, 6 HR. AFTER GUANIDINE (MG. PER 100 C.C.)
11	M.L.D.	No	No	3	27	54
12	M.L.D.	Yes	No	9	75	75
8	M.L.D.	No	Yes	3	38	65

Single doses of 1 mg. of glycine per gram of body weight were given by stomach tube to healthy guinea pigs that had fasted twenty-four hours. One hour later, the M.L.D. of guanidine hydrochloride was administered intramuscularly to 6 of the guinea pigs. The M.L.D. of methylguanidine sulfate was administered intramuscularly to the remaining 6 guinea pigs. All the guinea pigs showed the usual symptoms of guanidine intoxication and the majority died within twenty-four hours. Glycine in single doses, therefore, has no protective action against the M.L.D. of toxic guanidine salts.

The protective action of daily doses of glycine was then studied. Twenty-four guinea pigs were divided into two groups. Group A received daily doses of 1 mg. of glycine per gram of body weight. Group B received no glycine. On the second and following days, one-third M.L.D. of guanidine hydrochloride was administered intramuscularly to 4 guinea pigs of each group, one-third M.L.D. of methylguanidine sulfate was administered intramuscularly to an-

other 4 guinea pigs of each group, and one-third M.L.D. of methylguanidine sulfate was administered orally to the remaining 4 guinea pigs of each group. The amount of guanidine salt in each dose was uniformly increased at regular intervals until four-fifths M.L.D. was being given daily.

The average life of the guinea pigs receiving only the guanidine salts was twenty-eight days; the average life of those receiving glycine in addition was thirty-five days. On long-continued administration of guanidine salts, the guinea pigs appeared to develop a greater tolerance to the drug. However, the guinea pigs receiving glycine showed fewer and milder symptoms of guanidine intoxication and in addition lived longer. It appeared, therefore, that administered glycine aided the animals in overcoming the effects of toxic guanidine salts.

PROTECTIVE ACTION OF GLUCOSE

Watanabe[8] and others found that a severe hypoglycemia developed after the administration of toxic guanidine salts. Frank, Nothmann, and Wagner[9] found that the severity of spasms and the duration of life of animals poisoned by guanidine were dependent upon the amount of carbohydrate available in their organisms. Hummel[10] also noted this protective action of glucose, but Bakucz[11] was not convinced of its effect.

Experiments similar to those above were performed to study the protective action of glucose. The effects of single doses of glucose and of glycine against the M.L.D. of guanidine hydrochloride are shown in Table II. In experiments with daily administration, the average life of the guinea pigs receiving guanidine hydrochloride alone was eighteen days; of the guinea pigs receiving guanidine hydrochloride and glucose, nineteen days; and of the guinea pigs receiving guanidine hydrochloride and glycine, twenty-seven days. Glucose appeared to have no protective action.

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE URINE AND MUSCLE CHEMISTRY OF NORMAL GUINEA PIGS

The urine and muscles of guinea pigs were analyzed to serve as controls for comparison with the work to follow on dystrophic guinea pigs. Healthy guinea pigs were placed in small metabolism cages and given a diet of carrots, grain, water, and orange juice. The urine was collected under toluene, measured and filtered daily. The muscles were obtained at death or on sacrificing the animal.

Urine was collected for a ten-day period to serve as a normal. Treatment was then given as shown in Table III. One milligram of glycine per gram of body weight was administered daily at 10 A.M. and 4 P.M. One-third M.L.D. of guanidine hydrochloride was administered at 5 P.M. The amount of guanidine salt in each dose was uniformly increased at regular intervals, as in previous experiments, until four-fifths M.L.D. was being administered daily.

The urines were analyzed for creatine and creatinine,[12] guanidine,[13] and total acid-soluble phosphorus.[14] The results were averaged for the normal period and for the period in which treatment was given. The fresh gastroc-

nemius and gluteus muscles were analyzed for creatine,[15] creatinine,[12] and the acid-soluble phosphorus fractions.[14, 16, 17] The results were averaged and are shown in Table III.

TABLE III

URINE AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO NORMAL GUINEA PIGS

NO. OF GUINEA PIGS	4	4		4		4	
TREATMENT	NORMAL CONTROLS	NORMAL PERIOD	GLYCINE ADMINISTERED	NORMAL PERIOD	GUANIDINE ADMINISTERED	NORMAL PERIOD	GLYCINE AND GUANIDINE ADMINISTERED
Average days of treatment	42	10	32	10	26	10	32
Status at end of experiment	Alive		Alive		Dead		Dead
Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)							
Creatine	1.5	1.5	1.9	1.6	1.8	1.6	2.1
Creatinine	2.9	3.0	3.0	3.1	3.0	3.1	3.1
Guanidine base	0.5	0.5	0.5	0.5	3.8	0.5	3.2
Total acid-soluble phosphorus	3.6	3.5	3.6	3.7	4.0	3.6	3.8
Per cent administered guanidine recovered	--	--	--	--	39	--	31
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)							
Creatine	533	--	597	--	406	--	500
Creatinine	4.7	--	4.8	--	4.2	--	4.4
Acid-soluble phosphorus							
Total	182	--	161	--	116	--	146
Creatine phosphate	69	--	66	--	28	--	48
(per cent of total P)	(38)	--	(41)	--	(24)	--	(33)
Free orthophosphate	23	--	19	--	24	--	30
(per cent of total P)	(12)	--	(12)	--	(21)	--	(20)
Adenosine triphosphate	74	--	64	--	49	--	52
(per cent of total P)	(41)	--	(40)	--	(42)	--	(36)
Soluble esters	16	--	12	--	15	--	16
(per cent of total P)	(9)	--	(7)	--	(13)	--	(11)
Water content (%)	76.8	--	76.1	--	75.8	--	76.3

On the administration of glycine alone to normal guinea pigs, the excretion of creatine was increased, probably due to an increased production. An increased concentration of creatine was found in the muscles, although the relative per cent of creatine phosphate changed but little from the normal.

On the administration of guanidine hydrochloride alone to normal guinea pigs, an average of 39 per cent of the guanidine hydrochloride was recovered from the urine. The per cent of guanidine bases excreted decreased as the guanidine hydrochloride administered was increased. There may have been a maximum amount of guanidine that the kidney was capable of excreting or the decreased excretion may have been due to kidney damage. Histologic section of the kidneys showed some damage to the tubules. Some of the guani-

dine may have been converted to creatine, as was suggested by the slight increase in the excretion of creatine. It was more probable, however, that the guanidine hydrochloride either injured the muscle cell or made it more permeable, allowing an excessive loss of creatine to occur, as shown by the low concentration of creatine in the muscle. This loss may have stimulated the muscle or the centers of creatine formation to a greater production of creatine, with a subsequent further loss due to the abnormal permeability of the cell. This suggestion of an increased permeability was further supported by the loss of phosphates from the muscle and the decrease in the relative per cent of creatine phosphate.

On the administration of glycine with the guanidine hydrochloride, the excretion of creatine was increased, the effect being greater than when either glycine or guanidine hydrochloride were given alone. An average of 31 per cent of the guanidine hydrochloride administered was recovered from the urine. This was less than where the guanidine hydrochloride was given alone, and indicated that the glycine probably aided in the detoxication of the guanidine hydrochloride. This detoxication prevented the profound action of the guanidine hydrochloride on the muscle, as shown by the improved muscle metabolism and by the analysis of the muscle. The creatine and total phosphorus of the muscle and the relative per cent of creatine phosphate more nearly approached the normal.

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE URINE AND MUSCLE CHEMISTRY OF GUINEA PIGS WITH NUTRITIONAL MUSCULAR DYSTROPHY

Goettsch and Pappenheimer[15] found that a diet composed of rolled oats, wheat bran, casein, lard, cod-liver oil, sodium chloride, and calcium carbonate, treated with ethereal ferric chloride, to which was then added skimmed milk powder, led to a progressive, highly selective and ultimately fatal dystrophy of the voluntary muscles. They found guinea pigs and rabbits to be susceptible and rats to be resistant. This diet is complete in known requirements except for vitamin E. However, Goettsch and Pappenheimer found that the addition of this factor did not prevent the development of the disease. The lesions were not due to inanition, infection, or scurvy, and must be referred to some still unknown factor.

A muscular dystrophy was developed in several litters of guinea pigs. Visible symptoms of muscular dystrophy were shown by the sluggish actions of the guinea pigs, difficulty in righting themselves when placed on their backs, inability to use their hind legs, and finally, inability to rise when placed on their sides. When definite symptoms of muscular dystrophy appeared, the weight had reached a plateau and in most cases was decreasing.

As previously noted, there occurs an abnormal metabolism of creatine in certain muscular dystrophies. Accordingly, the effects of glycine and toxic guanidine salts were studied in dystrophic guinea pigs.

Thirty-one dystrophic guinea pigs were divided into 4 groups. The dystrophy diet was continued. Group 1 was given no additional treatment. Group 2 was given 1 mg. of glycine per gram of body weight daily. Group 3 was given one-third M.L.D. of guanidine hydrochloride daily. Group 4 was given both

glycine and guanidine hydrochloride according to the amounts previously noted. The amount of guanidine salt in each dose was uniformly increased at regular intervals, as in previous experiments. The experiment was continued to the death of the guinea pigs. The chronologic data are shown in Table IV.

Twelve dystrophic guinea pigs were divided into 4 groups. They were placed in small metabolism cages, and the dystrophy diet and orange juice were continued. The urine was collected under toluene, measured and filtered daily. Urine was collected for seven days after dystrophy developed to serve as a control period. Treatment was then given these 4 groups as detailed for the preceding 4 groups.

TABLE IV

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ADMINISTERED TO GUINEA PIGS WITH NUTRITIONAL MUSCULAR DYSTROPHY

TREATMENT	NO. OF GUINEA PIGS	AGE AT START OF DYSTROPHY (DAYS)	AGE AT TIME OF MARKED DYSTROPHY (DAYS)	INTERVAL FROM START TO MARKED DYSTROPHY (DAYS)	AGE AT DEATH (DAYS)	INTERVAL FROM START OF DYSTROPHY TO DEATH (DAYS)
None	7	94	103	9	110	16
Glycine	8	95	106	11	116	21
Guanidine hydrochloride	8	93	98	5	103	10
Glycine and Guanidine hydrochloride	8	97	105	8	112	15

The urines were analyzed as already detailed, and the results were averaged for each period of the experiment. The fresh gastrocnemius and gluteus muscles were analyzed as detailed. The results were averaged and are shown in Table V.

The guinea pigs in which a nutritional muscular dystrophy was developed excreted a larger amount of creatine than did normal guinea pigs. This may have been due to defective storage, to degeneration of the muscle tissue, or to a greater production. It is probable that all three mechanisms were involved. Dystrophic guinea pigs also excreted a smaller amount of creatinine as the dystrophy developed, probably due to the decreased creatine metabolism. Guanidine excretion was increased, probably due to defective metabolism of these compounds. The production of toxic guanidine compounds in the muscle cell may have lowered the permeability of the cell, thus accounting for the loss of creatine from the muscle and its increased excretion. An initial larger excretion of phosphorus was followed by a decreased excretion, indicating a probable loss of phosphorus compounds from the muscle. These findings were confirmed by the muscle analysis.

Glycine appeared to have some effect in delaying the progress of nutritional muscular dystrophy. Clinically, the animals appeared more normal and their span of life was increased. An increased excretion of creatine was noted and an increased concentration was found in the muscle. This increased concentration of creatine was responsible, probably, for the better functioning of the muscle and for the delay in the progress of the dystrophy.

The toxic effects of guanidine hydrochloride were more pronounced in dystrophic guinea pigs than in the normal. Glycine appeared again to reduce

its toxicity. On the administration of guanidine hydrochloride to dystrophic guinea pigs, an average of 63 per cent of the guanidine hydrochloride was recovered from the urine. These figures were markedly higher than those obtained with normal guinea pigs, indicating a defect in the metabolism and detoxication of these substances.

TABLE V

URINE AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO GUINEA PIGS WITH MUSCULAR DYSTROPHY

NO. OF GUINEA PIGS	3		3		3		3		12
TREATMENT	FIRST WEEK OF DYSTROPHY	BALANCE OF LIFE	FIRST WEEK OF DYSTROPHY	GLYCINE ADMINISTERED	FIRST WEEK OF DYSTROPHY	GUANIDINE ADMINISTERED	FIRST WEEK OF DYSTROPHY	GLYCINE AND GUANIDINE ADMINISTERED	AVERAGE OF ALL GUINEA PIGS FOR FIRST WEEK OF DYSTROPHY
Average days of treatment	7	9	7	14	7	5	7	11	7
Status at end of experiment		Dead		Dead		Dead		Dead	
Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)									
Creatine	2.3	3.7	2.6	4.2	2.8	5.0	2.4	4.4	2.5
Creatinine	3.4	2.9	2.8	2.7	2.9	2.5	2.8	2.6	3.0
Guanidine base	0.5	0.6	0.6	0.6	0.5	3.9	0.6	4.8	0.6
Total acid-soluble phosphorus	3.7	3.0	3.5	3.1	3.0	2.3	3.1	2.9	3.3
Per cent administered guanidine recovered	–	–	–	–	–	63	–	53	–
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)									
Creatine	–	206	–	248	–	151	–	190	–
Creatinine	–	1.8	–	2.1	–	1.6	–	2.0	–
Acid-soluble phosphorus									
Total	–	76	–	88	–	47	–	66	–
Creatine phosphate	–	23	–	32	–	11	–	20	–
(per cent of total P)	–	(30)	–	(36)	–	(23)	–	(30)	–
Free orthophosphate	–	15	–	11	–	12	–	12	–
(per cent of total P)	–	(20)	–	(13)	–	(26)	–	(18)	–
Adenosine triphosphate	–	29	–	35	–	16	–	25	–
(per cent of total P)	–	(38)	–	(40)	–	(34)	–	(38)	–
Soluble esters	–	9	–	10	–	8	–	9	–
(per cent of total P)	–	(12)	–	(11)	–	(17)	–	(14)	–
Water content (%)	–	76.0	–	76.4	–	75.7	–	76.1	–

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE BLOOD, URINE, AND MUSCLE CHEMISTRY OF NORMAL DOGS

In the previous experiments the small size of the guinea pigs prevented extensive blood, urine, and muscle examinations. Dogs were adopted, therefore, as the test animal. Guanidine hydrochloride was used since it showed a greater toxicity than did methylguanidine sulfate. The compound was administered intramuscularly as it exerted a more profound effect in this manner.

The blood, urine, and muscles of 16 dogs were analyzed to serve as controls for comparison with the work to follow on dystrophic dogs. Healthy dogs were placed in metabolism cages and given the normal diet of meat and meal, with water ad lib. They were weighed twice a week. Urine was collected under toluene, measured and filtered daily. Blood was drawn twice a week. The muscles were obtained at death or on sacrificing the animal. Urine and blood were collected for a period of two weeks to serve as a normal. Treatment was then given as shown in Table VI.

One milligram of glycine per gram of body weight was administered daily by capsule. Eight-tenths milligram of guanidine hydrochloride per gram of body weight was injected intramuscularly one hour after the glycine. After three weeks the glycine and guanidine were increased to one and one-half times the original dose; after another two weeks, to two times the original dose; and after another two weeks, to two and one-half times the original dose. This latter dosage was continued to the death of the animals receiving guanidine hydrochloride.

The dogs receiving guanidine hydrochloride alone showed signs of guanidine intoxication after about the second week. They were nervous and showed trembling, which passed on to tetanic twitchings, convulsions, and death. The average life of these dogs was twenty-four days. The dogs receiving both glycine and guanidine hydrochloride were nervous and showed trembling and tetanic twitchings only two to three days before death. There were no other signs of intoxication over the period of the experiment. The average life of these dogs was forty-seven days.

The blood specimens were analyzed for sugar,[19] nonprotein nitrogen,[20, 21] creatine and creatinine,[22] amino acids,[23] guanidine,[24] inorganic and total acid-soluble phosphorus,[14] and lactic acid.[21, 25] The urines were analyzed for total nitrogen,[20, 21] creatine and creatinine,[12] guanidine,[13] and inorganic and total acid-soluble phosphorus.[14] The results were averaged for each period of the experiment. Table VI shows the average findings during the normal period and during the period of maximum dosage where the most striking changes were noted. Mixed samples of gastrocnemius, anterior tibialis, and biceps and quadriceps femoris were analyzed for creatine,[15] creatinine,[12] guanidine,[13, 26] acid-soluble phosphorus fractions,[14, 16, 17] sodium,[27, 28] potassium,[29] calcium,[27] chlorine by a modified silver nitrate and thiocyanate titration, and water content by drying to constant weight. The average results are shown in Table VI. Muscle and kidney specimens were examined histologically.

The muscles of the normal dogs and those receiving glycine were grossly and microscopically normal. The muscles of the dogs receiving guanidine hydrochloride alone showed many swollen and granular areas with a marked nuclear reaction. The muscles of the dogs receiving glycine and guanidine hydrochloride were normal grossly. Microscopically, a few swollen fibers were seen showing a marked nuclear reaction. The kidneys showed some damage to the tubules.

The oral administration of glycine to normal dogs produced an increased excretion of creatine. No other change was noted other than the increased blood amino acid concentration and an increased excretion of nitrogen which were to be expected.

TABLE VI

BLOOD, URINE, AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO NORMAL DOGS

NO. OF DOGS	4	4		4		4	
TREATMENT	NORMAL CONTROLS	NORMAL PERIOD	GLYCINE ADMINISTERED	NORMAL PERIOD	GUANIDINE ADMINISTERED	NORMAL PERIOD	GLYCINE AND GUANIDINE ADMINISTERED
Average days of treatment	62	14	48	14	24	14	47
Status at end of experiment	Alive		Alive		Dead		Dead
Average Blood Chemistry (mg. per 100 c.c.)							
Sugar	84	82	83	86	66	84	75
Nonprotein nitrogen	28	26	27	29	48	28	54
Creatine	3.1	3.1	3.3	3.1	4.5	3.1	3.8
Creatinine	1.4	1.4	1.8	1.3	2.6	1.6	2.6
Amino acids	7.9	7.6	9.4	8.2	6.8	7.7	9.5
Guanidine base	0.4	0.3	0.3	0.4	2.5	0.4	2.9
Inorganic phosphorus	7.0	7.2	6.6	6.0	6.3	7.6	6.4
Total acid-soluble phosphorus	36	41	36	30	34	40	34
Lactic acid	17	17	18	16	37	17	29
Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)							
Total nitrogen	38	36	48	39	45	37	46
Creatine	1.0	0.9	1.4	1.0	1.3	0.9	1.2
Creatinine	2.5	2.2	2.3	2.7	2.6	2.4	2.6
Guanidine base	0.6	0.6	0.5	0.6	3.3	0.6	2.8
Inorganic phosphorus	2.5	2.3	2.6	2.6	2.7	2.4	2.8
Total acid-soluble phosphorus	4.5	4.5	4.7	4.7	4.8	4.5	4.4
Per cent administered guanidine recovered	-	-	-	-	28	-	23
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)							
Creatine	428	-	431	-	313	-	406
Creatinine	4.9	-	5.1	-	4.2	-	5.1
Guanidine base	0.7	-	0.7	-	2.4	-	1.8
Acid-soluble phosphorus							
Total	214	-	225	-	187	-	209
Creatine phosphate	87	-	92	-	42	-	72
(% of total P)	(41)	-	(41)	-	(23)	-	(34)
Free orthophosphate	28	-	32	-	49	-	35
(% of total P)	(13)	-	(14)	-	(26)	-	(17)
Adenosine triphosphate	88	-	90	-	68	-	81
(% of total P)	(41)	-	(40)	-	(36)	-	(39)
Soluble esters	11	-	11	-	28	-	21
(% of total P)	(5)	-	(5)	-	(15)	-	(10)
Sodium	43	-	40	-	82	-	57
Potassium	319	-	306	-	271	-	285
Calcium	8	-	9	-	6	-	8
Chlorine	62	-	60	-	114	-	77
Water content (%)	77.7	-	77.5	-	76.2	-	77.1

The administration of guanidine hydrochloride to normal dogs produced an increased excretion of creatine. The greatest rate of excretion was found to occur in the first week or two of guanidine administration. It was probable, therefore, that the guanidine injured the muscle cell or lowered its permeability, allowing creatine to diffuse more rapidly than normal. The muscle was found to have a decreased creatine content, a decrease in the total acid-soluble phosphorus, and both a relative and an absolute decrease of creatine phosphate. A decrease in the blood sugar and an increase in the lactic acid showed a disturbance of the carbohydrate metabolism. An increased nitrogen retention was noted. A decrease in the potassium and an increase in the sodium and chlorine of the muscle showed a profound disturbance in the electrolyte balance. Guanidine bases were increased throughout, due to their administration.

The administration of glycine with the guanidine reduced the toxic action of the latter. The large supply of glycine aided directly in the detoxication and also promoted the production of creatine to replace that lost from the muscles. Metabolism in general appeared to be more normal. A nitrogen retention was still present, but less severe disturbances of carbohydrate metabolism and electrolyte balance were noted.

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE BLOOD, URINE, AND MUSCLE CHEMISTRY OF DOGS WITH NUTRITIONAL MUSCULAR DYSTROPHY

A partial nutritional muscular dystrophy was developed in dogs. The dogs were weaned when they were forty-two days old and were placed in metabolism cages. The diet of Goettsch and Pappenheimer[18] was used. Sufficient water was added to the food to make a moist mass. This diet was supplemented by the use of additional skimmed milk powder dissolved in water, with the addition of vitamins A, C, and D. Water was allowed ad lib.

Morgulis and Spencer[30] found that the development of a nutritional muscular dystrophy following the diet of Goettsch and Pappenheimer could be prevented by the addition of whole wheat germ to the dystrophy-producing diet. Two dogs were placed on the diet with 10 per cent wheat germ added.

The dogs were weighed, and blood and urine were collected at two-week intervals during the first part of the experiment.

Visible symptoms of muscular dystrophy did not appear as rapidly nor was the dystrophy as pronounced as in the guinea pig. In about five months the weight had reached a plateau. Less interest was shown in food and the dogs appeared listless much of the time. A definite weakness finally appeared in the hind legs. Many of the animals died of pneumonia. These dogs were not included in the average findings given here.

When definite signs of weakness appeared in the hind legs, the dogs were weighed twice a week, urine was collected under toluene daily, blood was drawn twice a week, and the muscles were obtained at death or on sacrificing the animal. Treatment was then instituted as noted in Table VII.

The same amounts of glycine and guanidine were given as detailed above. After three weeks they were regularly increased as previously noted.

Blood and urine specimens were analyzed as previously discussed and the results were averaged for each period of the experiment. Table VII shows

TABLE VII

BLOOD, URINE, AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO DOGS WITH MUSCULAR DYSTROPHY

NO. OF DOGS	4			2			2			2			2
TREATMENT	DYSTROPHY CONTROLS			GLYCINE ADDED			GUANIDINE ADDED			GLYCINE AND GUANIDINE ADDED			WHEAT GERM ADDED
	DYST. DIET	DYST. DIET	DYST. DIET	DYST. DIET	PLUS GLYCINE	PLUS GLYCINE	DYST. DIET	PLUS GUANIDINE	PLUS GUANIDINE	DYST. DIET	PLUS GLYCINE AND GUANIDINE	PLUS GLYCINE AND GUANIDINE	DYST. DIET PLUS 10% WHEAT GERM
Clinical State	Norm.	Sl. dyst.	Mkd. dyst.	Norm.	Sl. dyst.	Mkd. dyst.	Norm.	Sl. dyst.	Mkd. dyst.	Norm.	Sl. dyst.	Mkd. dyst.	Norm.
Average days of treatment	161	7	19	168	26	21	154	3	10	159	12	18	215
Status at end of experiment			Dead			Dead			Dead			Dead	Alive
Average Blood Chemistry (mg. per 100 c.c.)													
Sugar	78	75	60	76	85	73	73	61	51	76	87	73	85
Nonprotein nitrogen	25	28	43	27	33	42	30	41	49	28	32	47	27
Creatine	3.0	3.0	2.3	3.0	3.2	2.8	2.9	3.5	3.8	3.0	3.4	2.8	3.1
Creatinine	1.5	1.1	1.0	1.6	1.7	1.3	1.5	1.8	1.7	1.7	2.0	2.0	1.4
Amino acids	9.5	9.7	8.3	8.5	9.5	8.7	9.2	8.4	8.1	8.7	9.7	8.8	8.9
Guanidine base	0.4	0.6	0.6	0.4	0.5	0.5	0.4	2.2	2.6	0.4	0.7	1.4	0.3
Inorganic phosphorus	8.1	7.7	8.8	8.8	8.8	8.0	9.0	8.1	7.7	7.6	8.4	7.6	8.4
Total acid-soluble phosphorus	56	54	60	50	55	48	48	49	41	50	55	50	49
Lactic acid	19	21	17	19	20	19	20	32	34	18	21	28	18

Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)													
Total nitrogen	94	88	95	95	105	110	84	92	97	90	100	103	84
Creatine	1.5	1.6	2.4	1.2	1.6	2.0	1.1	1.4	1.7	1.0	1.1	1.7	1.1
Creatinine	2.2	1.9	1.7	2.3	2.7	3.2	2.4	2.2	1.8	2.1	2.4	2.9	2.5
Guanidine base	0.7	0.8	0.9	0.6	0.6	0.7	0.8	1.8	2.5	0.5	1.0	2.5	0.6
Inorganic phosphorus	2.4	2.8	3.1	2.2	2.8	2.8	2.7	2.5	2.2	3.0	2.9	3.0	2.8
Total acid-soluble phosphorus	5.1	5.3	4.1	4.8	5.4	5.1	5.5	5.5	5.0	5.8	6.3	6.3	5.3
Per cent administered guanidine recovered	–	–	–	–	–	–	–	48	59	–	40	48	–
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)													
Creatine	–	–	311	–	–	343	–	–	263	–	–	332	395
Creatinine	–	–	3.4	–	–	3.7	–	–	3.1	–	–	3.7	4.7
Guanidine base	–	–	1.1	–	–	0.9	–	–	2.9	–	–	1.4	0.6
Acid-soluble phosphorus													
Total	–	–	144	–	–	152	–	–	126	–	–	140	192
Creatine phosphate	–	–	46	–	–	58	–	–	31	–	–	45	78
(% of total P)	–	–	(32)	–	–	(38)	–	–	(25)	–	–	(32)	(41)
Free orthophosphate	–	–	27	–	–	20	–	–	31	–	–	29	23
(% of total P)	–	–	(19)	–	–	(13)	–	–	(26)	–	–	(21)	(12)
Adenosine triphosphate	–	–	55	–	–	59	–	–	43	–	–	51	81
(% of total P)	–	–	(38)	–	–	(39)	–	–	(34)	–	–	(36)	(42)
Soluble esters	–	–	16	–	–	15	–	–	19	–	–	15	10
(% of total P)	–	–	(11)	–	–	(10)	–	–	(15)	–	–	(11)	(5)
Sodium	–	–	90	–	–	61	–	–	112	–	–	87	45
Potassium	–	–	237	–	–	280	–	–	197	–	–	244	323
Calcium	–	–	32	–	–	15	–	–	18	–	–	23	9
Chlorine	–	–	117	–	–	101	–	–	122	–	–	112	71
Water content (%)	–	–	76.1	–	–	76.7	–	–	75.8	–	–	76.0	77.5

the average findings for the dogs in each group during the period when they were on the dystrophy diet alone and appeared to be normal clinically; during the period of slight dystrophy, as shown by slight weakness; and during the period of marked dystrophy, as shown by marked weakness before death. The muscle specimens were analyzed as already described, and histologic sections were made. The results of the chemical analyses are shown in Table VII.

At autopsy the muscles of the thighs and hind legs of the dogs, which showed marked weakness before death, were found to be pale in color and flabby. Areas of damaged fibers were seen. Many were swollen, showing degeneration and a marked nuclear reaction. A moderate infiltration with fibrous tissue was seen. The muscles of the dogs receiving 10 per cent wheat germ were normal.

Little metabolic change was noted until the muscular dystrophy became evident clinically. A slight increase in the excretion of creatine was noted, seen more especially in the daily analyses than in the averages shown. When marked muscular dystrophy developed, a further increase was noted in the excretion of creatine, accompanied by an increased excretion of total acid-soluble phosphorus. A low blood creatine concentration finally developed and a low concentration of creatine and creatine phosphate were found in the muscles. An increased concentration of guanidine base was found in the blood, urine, and muscle. The increased concentration of guanidine in the muscle may have produced an increased permeability, with the resulting loss of creatine and phosphate. The increased guanidine was due, probably, to some defect in the metabolism of guanidine compounds. A disturbance also was seen in the carbohydrate and mineral metabolism, similar to that seen in the normal dogs when toxic guanidine compounds were injected intramuscularly.

Glycine appeared to delay the effects of the nutritional muscular dystrophy, as shown by the blood, urine, and muscle chemistry. The animals lived longer and their metabolism was more normal. The basis of this improvement may have been the increased concentration of creatine in the muscle due to its increased production.

Guanidine hydrochloride showed a greater toxicity in dogs suffering from a nutritional muscular dystrophy than it did in normal dogs. Glycine again aided in the detoxication of this added guanidine.

DISCUSSION

The administration of toxic guanidine salts over a period of several weeks results in a loss of creatine and phosphate from voluntary muscle. It appears probable that guanidine increases the permeability of the muscle cell, allowing creatine and phosphate to diffuse more rapidly than normal when they have been separated during muscular action. This suggestion is supported by the work of Gavrilescu,[31] who found by conductivity experiments that guanidine compounds increased the permeability of abdominal muscles. The lowered concentration of creatine in the muscle may stimulate the centers of creatine production, resulting in an increased production and further loss of creatine.

Increased blood guanidine and increased guanidine excretion have been noted in many conditions. Much confusion has resulted from the attempt to

associate various clinical symptoms with the level of blood guanidine. It is suggested that blood guanidine is the result, and not the cause, of endogenous guanidine intoxication. A guanidine derivative produced in any tissue may exert a more profound action in that tissue than it may exert after diffusion into the extracellular body fluids. The level of the blood guanidine probably depends on the activity of the centers of detoxication.

In exogenous guanidine intoxication other physiologic effects of guanidine may overshadow its action on the muscle cell. This effect is increased by the apparent ability of the animal to acclimate itself to larger amounts of administered guanidine over a period of time.

There is a similarity in the blood, urine, and muscle chemistry of guanidine intoxication and of nutritional muscular dystrophy. The increased concentration of simple guanidine compounds in nutritional muscular dystrophy suggests the possibility that these compounds may be the cause of the metabolic disturbance in this condition. The increased concentration of guanidine in the blood and urine of patients with progressive muscular dystrophy, as found by MacFate,[32] Sullivan, Hess, and Irrevere[33] and others, and the similarity of the muscle findings in this paper to the findings in progressive muscular dystrophy, as reported by Nevin,[17] Reinhold and Kingsley,[34] and others, suggest the possibility that toxic guanidine compounds may be responsible for, or at least initiate, the abnormal processes found in progressive muscular dystrophy and other dystrophies accompanied by like metabolic changes.

Hines and Knowlton[35] believe that the findings of altered concentration of various substances in atrophic muscles can be attributed to relative changes in the amounts of muscle cell and nonmuscle cell phases rather than to a change in the composition of the various phases. They found the increase in chloride concentration of atrophied muscle (from denervation, fasting, and tenotomy) to parallel the extent of atrophy and the relative increase in connective tissue, and, therefore, concluded that the chlorides of the muscle are chiefly associated with the connective tissue. Morgulis and Osheroff[36] state that at least a part of the chloride is a component of the connective tissues and that the increase in chloride content of dystrophic muscles is undoubtedly associated with the increase in fibrous tissue. The moderate increase in connective tissue noted in these experiments may account, in some degree, for the chemical changes observed. However, microscopic study did not reveal as great an increase in the connective tissue as is usually seen in cases of atrophy from denervation, fasting, and tenotomy. Further, because of the rapid and early chemical changes noted, it is believed that some other factor, such as an increased permeability of the muscle cell, must be considered.

The therapeutic value of glycine in these conditions probably lies in its stimulation of an increased production of creatine. This may be due to the specific dynamic action of the glycine itself (Rapport and Katz[37]). An increased glycine concentration in the blood and body fluids may stimulate the production of large amounts of creatine until the creatine production centers adjust themselves to this new and increased level of glycine. The rate of creatine production may then drop to approximately normal, accounting for the decreased excretion of creatine found in many patients after glycine therapy

over a long period of time, as reported by Thomas, Milhorat, and Techner,[4] Milhorat,[5] and others. The value of glycine in the treatment of progressive muscular dystrophy apparently depends upon the ability of the muscle cell to utilize the creatine produced. This ability appears to be lost gradually as the muscular dystrophy progresses.

The toxicity of guanidine derivatives varies greatly (Alles[35]). The general finding of increased blood guanidine cannot be properly evaluated until methods are perfected for determining the exact guanidine derivative present.

CONCLUSIONS

1. The daily administration of one-third to four-fifths M.L.D. of a toxic guanidine salt, over a period of several weeks, produces changes in the metabolism and structure of the muscle, similar to those found in nutritional muscular dystrophy and in progressive muscular dystrophy. There are produced a loss of creatine and phosphate from the muscle and severe disturbances in carbohydrate metabolism and the electrolyte balance. It is suggested that guanidine may lower the permeability of the muscle cell, producing these changes.

2. One milligram of glycine per gram of body weight, administered daily, appears to afford some protection against small daily doses (one-third to four-fifths M.L.D.) of a toxic guanidine salt.

3. Glycine delays the progress of a nutritional muscular dystrophy.

4. It is suggested that the abnormal metabolism of guanidine compounds may be an etiologic factor in the production of a progressive muscular dystrophy.

REFERENCES

1. Levene, P. A., and Kristeller, L.: Factors Regulating the Creatinine Output in Man, Am. J. Physiol. 24: 45, 1909.
2. Brand, E., Harris, M. M., Sandberg, M., and Ringer, A. I.: Studies on the Origin of Creatine, Am. J. Physiol. 90: 296, 1929.
3. Magee, M. C.: Creatine and Creatinine Metabolism in Progressive Muscular Dystrophy, Am. J. Dis. Child. 43: 19, 1932.
4. Thomas, K., Milhorat, A. T., and Techner, F.: Untersuchungen über die Herkunft des Kreatins. Ein Beitrag zur Behandlung progressiver Muskelatrophien mit Glykokoll, Ztschr. f. physiol. Chem. 205: 93, 1932; Weitere Untersuchungen über die Herkunft des Kreatins, Ztschr. f. physiol. Chem. 214: 121, 1933.
5. Milhorat, A. T.: Über die Behandlung der progressiven Muskeldystrophie und ähnlicher Muskelerkrankungen mit Glykokoll, Deutsches Arch. f. klin. Med. 174: 487, 1933.
6. Tripoli, C. J., and Beard, H. H.: Muscular Dystrophy and Atrophy, Arch. Int. Med. 53: 435, 1934.
7. [illegible] and Bunge, G.: Arbeiten aus dem Laboratorium für experimentelle [illegible] zu Strassburg, Arch. f. exper. Path. u. Pharmakol. 6: 233, 1876.
8. Watanabe, C. K.: Studies in the Metabolic Changes Induced by Administration of Guanidine Bases, J. Biol. Chem. 34: 51, 1918.
9. Frank, E., Nothmann, M., and Wagner, A.: Über den Angriffspunkt des Insulins, Arch. f. exper. Path. u. Pharmakol. 110: 225, 1925.
10. Hummel, H.: Beitrag zur Bedeutung des Traubenzuckers für Entgiftungsvorgänge im Organismus, Klin. Wchnschr. 3: 1573, 1924.
11. Bakucz, J.: Beiträge zur Kenntnis der entgiftenden Wirkung des Traubenzuckers bei Guanidinvergiftung, Arch. f. exper. Path. u. Pharmakol. 110: 121, 1925.
12. Folin, O.: On the Determination of Creatinine and Creatine in Urine, J. Biol. Chem. 17: 469, 1914.
13. Andes, J. E., and Myers, V. C.: Colorimetric Estimation of Guanidine-like Substances in the Urine, J. Biol. Chem. 118: 137, 1937.
14. Youngburg, G. E., and Youngburg, M. Y.: A System of Blood Phosphorus Analysis, J. Lab. & Clin. Med. 16: 158, 1930.
15. Rose, W. C., Helmer, O. M., and Chanutin, A.: A Modified Method for the Estimation of Total Creatinine in Small Amounts of Tissues, J. Biol. Chem. 75: 543, 1927.

16. Eggleton, G. P., and Eggleton, P.: A Method of Estimating Phosphagen and Some Other Phosphorus Compounds in Muscle Tissue, J. Physiol. 68: 193, 1929.
17. Nevin, S.: A Study of the Muscle Chemistry in Myasthenia Gravis, Pseudohypertrophic Muscular Dystrophy and Myotonia, Brain 57: 239, 1934.
18. Goettsch, M., and Pappenheimer, A. M.: Nutritional Muscular Dystrophy in the Guinea Pig and Rabbit, J. Exper. Med. 54: 145, 1931.
19. Benedict, S. R.: Determination of Sugar in Blood, J. Biol. Chem. 83: 165, 1929.
20. Koch, F. C., and McMeekin, T. L.: A New Direct Nesslerization Micro-Kjeldahl Method and a Modification of the Nessler-Folin Reagent for Ammonia, J. Am. Chem. Soc. 46: 2066, 1924.
21. Levinson, S. A., and MacFate, R. P.: Clinical Laboratory Diagnosis, Philadelphia, 1937, Lea & Febiger.
22. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. 38: 81, 1919.
23. Folin, O.: A System of Blood Analysis, J. Biol. Chem. 51: 377, 1922.
24. Andes, J. E., and Myers, V. C.: Guanidine-like Substances in Blood, J. Lab. & Clin. Med. 22: 1147, 1937.
25. Von Fürth, O., and Charnass, D.: Über die quantitative Bestimmung der Milchsäure durch Ermittlung der daraus abspaltbaren Aldehydmenge, Biochem. Ztschr. 26: 199, 1910.
26. Ellis, M. M.: Guanidine Determinations on Some Invertebrates by a Colorimetric Phosphotungstic Acid Method, Biochem. J. 22: 353, 1928.
27. Wang, C. C.: Improvements in the Methods for Calcium Determination in Biological Material, J. Biol. Chem. 111: 443, 1935.
28. Salit, P. W.: A New Triple Acetate Method for Sodium Determinations in Biological Materials, J. Biol. Chem. 96: 659, 1932.
29. Kramer, B., and Tisdall, F. F.: The Direct Quantitative Determination of Sodium, Potassium, Calcium, and Magnesium in Small Amounts of Blood, J. Biol. Chem. 48: 223, 1921.
30. Morgulis, S., and Spencer, H. C.: A Study of the Dietary Factors Concerned in Nutritional Muscular Dystrophy, J. Nutrition 11: 573, 1936.
31. Gavrilescu, N.: L'action de la guanidine sur la permeabilite des muscles, Ann. de physiol. 5: 294, 1929.
32. MacFate, R. P.: Unpublished Reports From the Hospital Laboratories of the Research and Educational Hospitals, Chicago, Ill.
33. Sullivan, M. X., Hess, W. C., and Irrevere, F.: Studies in Muscular Dystrophy, J. Biol. Chem. 114: 633, 1936.
34. Reinhold, J. G., and Kingsley, G. R.: The Chemical Composition of Voluntary Muscle in Muscle Disease: A Comparison of Progressive Muscular Dystrophy With Other Diseases Together With a Study of Effects of Glycine and Creatine Therapy, J. Clin. Investigation 17: 377, 1938.
35. Hines, H. M., and Knowlton, G. C.: Electrolyte and Water Changes in Muscle During Atrophy, Am. J. Physiol. 120: 719, 1937.
36. Morgulis, S., and Osheroff, W.: Mineral Composition of the Muscles of Rabbits on a Diet Producing Muscle Dystrophy, J. Biol. Chem. 124: 767, 1938.
37. Rapport, D., and Katz, L. N.: The Effect of Glycine Upon the Metabolism of Isolated Perfused Muscle, Am. J. Physiol. 80: 185, 1927.
38. Alles, G. A.: The Comparative Physiological Action of Some Derivatives of Guanidine, J. Pharmacol. & Exper. Therap. 28: 251, 1926.

A COMPARATIVE STUDY OF DEXTROSE AND DEXTRIN TOLERANCE IN PATIENTS WITH CHRONIC ULCERATIVE COLITIS*

ROBERT C. PAGE, M.D., Z. BERCOVITZ, M.D., AND EDWIN J. DE BEER, PH.D.
NEW YORK, N. Y.

ABSORPTION studies in chronic ulcerative colitis have been confined mainly to dextrose, and it seemed of interest to us to study the intestinal absorption and the blood sugar response of another carbohydrate. For this study, a mixture of polysaccharides (dextrins and maltose) produced by the partial breakdown of starch was selected. For control purposes dextrose absorption was studied simultaneously. Tolerance curves served as a measure of the absorption process.

Low or flat blood sugar curves for oral dextrose tolerance tests have been frequently found by us (Z. B. and R. C.P.)[1] in patients with chronic ulcerative colitis. In one group of 58 standard oral, one dose, two-hour dextrose tolerance tests, 23 (39.6 per cent) tests were of the low or flat type. Groen,[2] using a simplified technique of intestinal intubation, ascribed the decreased absorption of dextrose to a defect in the absorptive capacity of the intestinal wall and not to an unusually rapid passage of the intestinal contents.

METHOD OF STUDY

Twenty-three patients with chronic ulcerative colitis attending an outpatient clinic were studied. All the patients had had the disease for periods of one or more years.

Both the oral dextrose tolerance test and the oral dextrin tolerance test were carried out on the same patient. These tests were alternated in successive patients. The tests were carried out from six to ten days of each other, with no change in diet, so that each patient served as his own control.

The patients reported in the morning without breakfast. They were weighed and a fasting venous blood sample was obtained. One gram per kilogram of body weight of dextrose or dextrin† in 150 to 200 c.c. water was given, and blood samples were taken at one-, two- and three-hour intervals, and in some instances at four and five hours. Urine was also collected and examined for dextrose at these intervals. The venous blood was analyzed for true dextrose content by the Shaffer-Somogyi[3] method. The results, therefore,

*From the Colitis Clinic, Department of Medicine, New York Post-Graduate Medical School and Hospital, New York.

Read at the Annual Meeting of the American Federation for Clinical Research, Minneapolis, Minn., April 20, 1942.

Received [illegible] ion, July 1, 1942.

†Since [illegible] s found unsuitable because of unpalatability and difficulty of making acceptab[illegible] Welleome & Co.), containing 75 per cent dextrins and 24 per [illegible] moisture, and 0.25 per cent mineral matter, was used. Since the p[illegible] polysaccharide and disaccharide combination consists of d[illegible] in the text to denote this dextrin-maltose preparation.

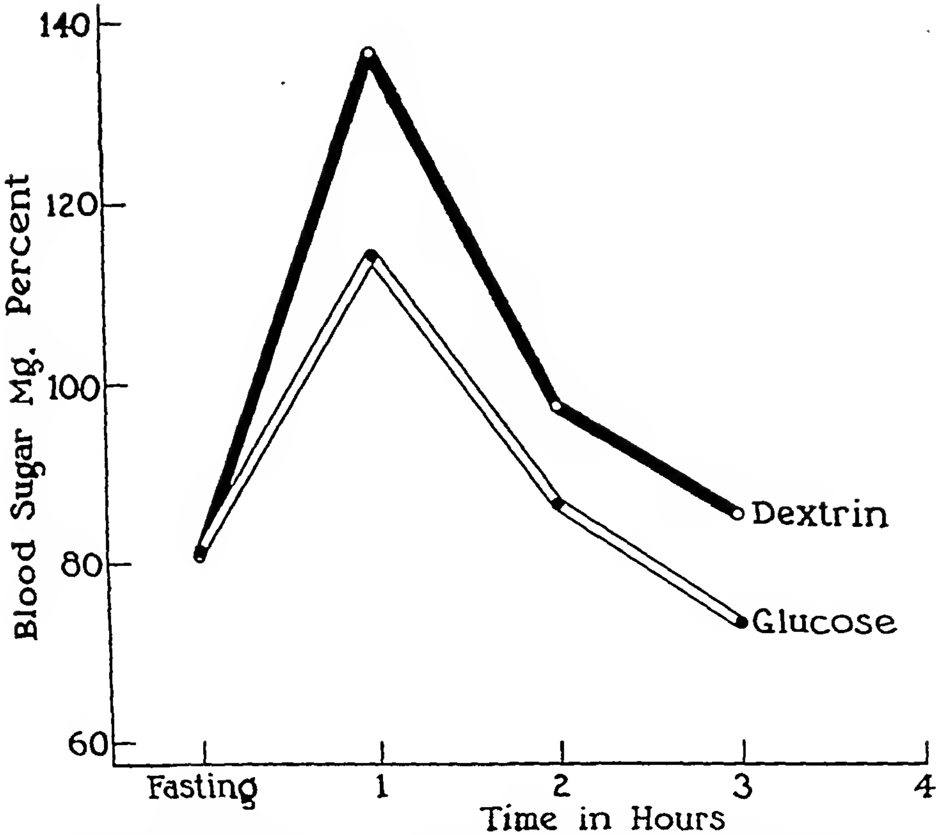


Fig. 1.—Graph showing mean values of comparative results of oral dextrose and dextrin tolerance tests on 23 patients with chronic ulcerative colitis, as given in Table I.

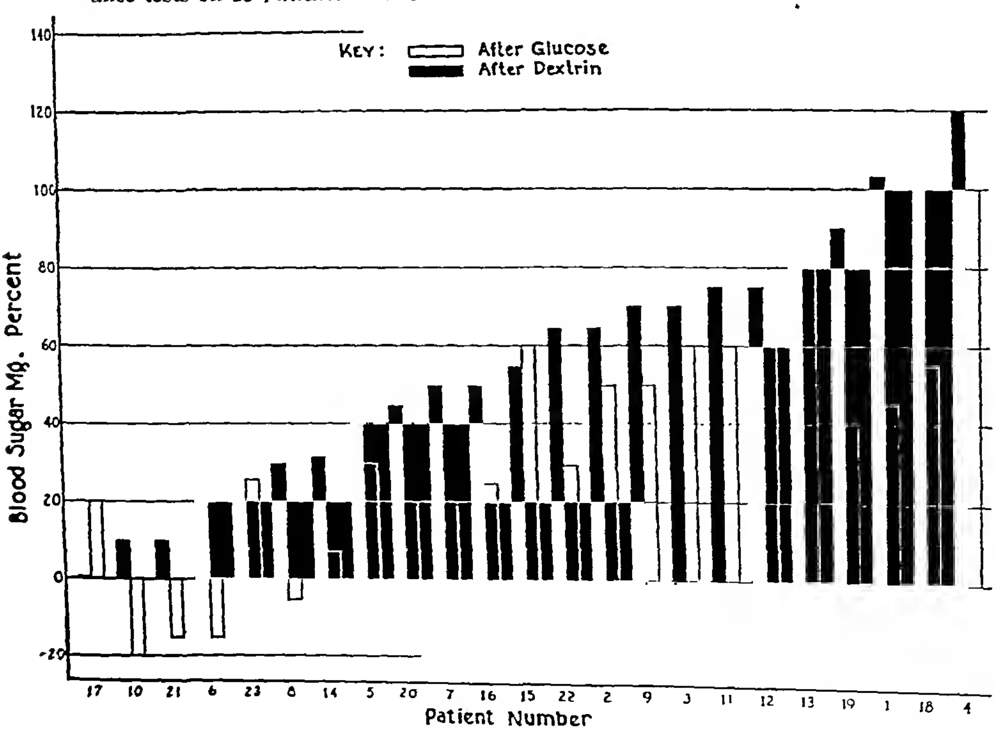


Fig. 2.—Bar graph showing comparison of increase or decrease in blood dextrose level from fasting level one hour after oral dextrose and dextrin tolerance tests in the same patients.

A COMPARATIVE STUDY OF DEXTROSE AND DEXTRIN TOLERANCE IN PATIENTS WITH CHRONIC ULCERATIVE COLITIS*

ROBERT C. PAGE, M.D., Z. BERCOVITZ, M.D., AND EDWIN J. DE BEER, PH.D.
NEW YORK, N. Y.

ABSORPTION studies in chronic ulcerative colitis have been confined mainly to dextrose, and it seemed of interest to us to study the intestinal absorption and the blood sugar response of another carbohydrate. For this study, a mixture of polysaccharides (dextrins and maltose) produced by the partial breakdown of starch was selected. For control purposes dextrose absorption was studied simultaneously. Tolerance curves served as a measure of the absorption process.

Low or flat blood sugar curves for oral dextrose tolerance tests have been frequently found by us (Z.B. and R.C.P.)[1] in patients with chronic ulcerative colitis. In one group of 58 standard oral, one dose, two-hour dextrose tolerance tests, 23 (39.6 per cent) tests were of the low or flat type. Groen,[2] using a simplified technique of intestinal intubation, ascribed the decreased absorption of dextrose to a defect in the absorptive capacity of the intestinal wall and not to an unusually rapid passage of the intestinal contents.

METHOD OF STUDY

Twenty-three patients with chronic ulcerative colitis attending an outpatient clinic were studied. All the patients had had the disease for periods of one or more years.

Both the oral dextrose tolerance test and the oral dextrin tolerance test were carried out on the same patient. These tests were alternated in successive patients. The tests were carried out from six to ten days of each other, with no change in diet, so that each patient served as his own control.

The patients reported in the morning without breakfast. They were weighed and a fasting venous blood sample was obtained. One gram per kilogram of body weight of dextrose or dextrin† in 150 to 200 c.c. water was given, and blood samples were taken at one-, two- and three-hour intervals, and in some instances at four and five hours. Urine was also collected and examined for dextrose at these intervals. The venous blood was analyzed for true dextrose content by the Shaffer-Somogyi[3] method. The results, therefore,

*From the Colitis Clinic, Department of Medicine, New York Post-Graduate Medical School and Hospital, New York.

Read at the Annual Meeting of the American Federation for Clinical Research, Minneapolis, Minn., April 20, 1942.

Received for publication, July 1, 1942.

†Since pure dextrin was found unsuitable because of unpalatability and difficulty of making acceptable solutions, "Dexin" (Burroughs Wellcome & Co.), containing 75 per cent dextrins and 24 per cent maltose, 0.75 per cent moisture, and 0.25 per cent mineral matter, was used. Since the predominant constituent of this polysaccharide and disaccharide combination consists of dextrin, we have used the term *dextrin* in the text to denote this dextrin-maltose preparation.

Urinalyses on the entire group of patients using quantitative Benedict's test revealed reducing substances in only one patient (No. 8). The fasting specimen was negative, and in the first-hour specimen after oral dextrose the reducing substances were present to the amount of 1.65 Gm., after the second hour 1.13 Gm., and after the third hour 0.45 Gm. In the dextrin test the fasting specimen was negative. After one hour the urine contained 0.59 Gm. of reducing substance, after the second hour 0.45 Gm., and after the third hour 0.19 Gm.

DISCUSSION OF RESULTS

The foregoing observations apparently indicate that at all points the blood sugar levels were higher after the dextrins than after the dextrose. However, it is well known that because of the many variables involved, blood sugar curves undergo wide fluctuations. Therefore, a statistical analysis of the results was made in order to determine whether the greater hyperglycemia following the dextrin administration was real or whether it was only an accident due to chance.

Statistical Analysis.—The dextrin and the dextrose curves were compared by calculating the statistic "t" for the differences in the blood sugar means at the one-hour, two-hour, and three-hour periods. The respective "t" values found were 5.568, 3.846, and 2.756, and the corresponding probabilities, as given by the tables of Fisher and Yates,[4] were $P = <0.001$, $P = <0.001$ and $P = >0.001$ but <0.01. Since a probability of $P = 0.001$ indicates one chance in 1,000 and a probability of $P = 0.01$ indicates one chance in 100, one may conclude the difference is real and that dextrin administration produced higher blood sugar values than did dextrose administration.

Since 100 mg. of the dextrin mixture are theoretically capable of giving approximately 108 mg. of dextrose if completely hydrolyzed, an approximate correction was made by reducing the mean difference by 8 per cent and again calculating the "t" values. This procedure did not appreciably alter the results.

One hundred milligrams of the dextrin preparation used in this study contained 75 mg. of dextrin, $(C_6H_{10}O_5)_n \cdot H_2O$, which if completely hydrolyzed would yield 83.3 mg. of dextrose (using the factor of 0.9, since nine parts of dextrin give ten parts of dextrose upon hydrolysis),[5] and 24 mg. of anhydrous maltose $(C_{12}H_{22}O_{11})$, which would yield 25.2 mg. of dextrose (using the factor of 0.95) if completely hydrolyzed, making a total of 108.5 mg. of dextrose. Actually the maltose is present in the hydrated state $(C_{12}H_{22}O_{11} \cdot H_2O)$ so that 24 mg., when hydrolyzed, yields 24 mg. of dextrose (1.2 mg. less than with anhydrous maltose). Using this figure in the calculation, 100 mg. of the dextrin-maltose mixture would yield 107.3 mg. of dextrose instead of 108.5 mg. We have compromised between these two values and assume that 100 mg. of the dextrin mixture would be converted into 108 mg. of dextrose, or an 8 per cent increase over the original weight.

The possibility exists that a comparison of the areas under the blood dextrose curves may present a more comprehensive picture than comparisons made separately at three different points. Accordingly, the areas under the 46 curves were measured with a polar planimeter. Areas below a base line passing through the fasting level were considered negative. These data were subjected to an analysis of variance following the standard procedure, such

represent dextrose content with only little interference from other reducing substances (vitamin C, glutathione, methionine, cysteine, etc.).

RESULTS OF STUDY

The results are given in Table I and are presented graphically in Figs. 1 and 2. It will be observed that after dextrose administration the blood sugar at the end of the first hour had risen sharply to an average value of 115 mg. per 100 c.c., an increase of approximately 42 per cent over the average fasting level of 81 mg. per 100 c.c. However, at the end of the second hour it had dropped sharply to 86, being then only 7 per cent above the fasting level. At the end of the third hour it had fallen to 73, or 10 per cent below the fasting level. At the end of the fourth hour the lowest value was reached, 69, or 15 per cent below the fasting level. The four determinations available at the fifth hour showed that blood sugar was starting to recover, now being 75, or 7 per cent below the fasting level.

TABLE I

PATIENT NO.	GLUCOSE						DEXTRIN					
	TRUE BLOOD GLUCOSE (MILLIGRAMS PER 100 C.C.)—SHAFFER-SOMOGYI METHOD											
	FASTING LEVEL	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	FASTING LEVEL	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.
1	70	115	110	85	80		105	208	190	135	110	
2	80	130	95	90	80	85	95	160	145	95	65	80
3	90	150	140	95	80	95	70	140	145	105	75	60
4	80	180	110	70			80	200	135	70		
5	85	115	100	55	55		70	110	65	60		
6	95	80	75	80			80	100	85	90		
7	65	85	60	90			60	110	100	90		
8	105	100	95	60			110	140	105	85		
9	80	130	135	115	85		80	150	160	100		
10	90	70	45	70			70	80	60	60		
11	70	130	70	60			85	160	80	70		
12	95	155	95	80			75	150	70	60		
13	70	150	75	60	70	75	70	150	85	65	65	
14	90	98	65	75	80		80	113	75	75		
15	70	130	65	60	60		80	135	85	75		
16	105	130	80	70			85	135	90	65		
17	65	85	55	45	55		70	70	75	75	60	
18	80	135	100	50			70	180	105	50		
19	55	95	80	45	45	45	65	155	85	140		
20	80	100	95	90	65		115	160	115	95	85	
21	100	85	60	85			80	90	50	85		
22	70	100	90	70			80	145	125	130		
23	70	95	90	80	70		75	95	105	85	75	
Means	80.9	114.9	86.3	73.0	68.8	75.0	80.4	136.4	97.2	85.2	76.4	70.0

After dextrin administration the blood sugar showed a similar but much greater rise by the end of the first hour. From the fasting level of 80 mg. per 100 c.c., the blood sugar rose to 136 at the end of the first hour, an increase of 70 per cent. By the end of the second hour the drop had not reached as low a level as with dextrose, the figure being 97, or 21 per cent above fasting. At the end of the third hour the blood sugar was 85, or still 6 per cent above the fasting level. The seven available observations at the end of the fourth hour gave 76.4, or a drop of 5 per cent below fasting level. The two fifth-hour observations averaged 70, or 13 per cent below fasting level.

HYPERBILIRUBINEMIA FOLLOWING ADMINISTRATION OF SULFONAMIDES*

A. Cantarow, M.D., and C. W. Wirts, M.D.†
Philadelphia, Pa.

THE present report is concerned with the occurrence of hyperbilirubinemia and other evidence of hepatic functional impairment during or after administration of sulfonamide compounds to twenty patients.

COMMENT

The pertinent data are presented in the table. Visible icterus was absent in six instances (Cases 1, 4, 12, 14, 15, 19). Total and direct-reacting bilirubin was determined by the method of Malloy and Evelyn.[1] The existence of hyperbilirubinemia was first noted one to seven days after institution of sulfonamide therapy, after administration of 6 to 24 Gm. of the drug, and with blood sulfonamide concentrations of 4 to 14 mg. per 100 c.c. Recovery occurred in every case.

It is generally stated that hepatocellular damage occurs not infrequently during administration of sulfanilamide,[2-9] seldom with sulfapyridine,[2] and rarely with sulfathiazole,[2] while it has not been observed following the use of sulfadiazine.[2, 10] In the present series, evidence of hepatic functional impairment, other than hyperbilirubinemia and excessive urobilinuria, was demonstrated in Cases 2, 6 (sulfanilamide), 10, 11, 13 (sulfathiazole), 15, 16 (sulfapyridine), 17, 18, and 20 (sulfadiazine). In Case 7 the hyperbilirubinemia was probably fundamentally of hemolytic origin, as suggested by the absence of bromsulfalein retention, the anemia, and the relatively low proportion of direct-reacting bilirubin in the serum. The same may be true of Cases 4 and 19. In Cases 1 and 2, 4 Gm. of sulfanilamide was placed in the peritoneum at the time of operation. It has been shown[6] that additional sulfonamide therapy following this procedure enhances the possibility of development of hepatitis. Because of the occasional development of hyperbilirubinemia and other evidence of hepatic functional impairment in patients with peritonitis and pneumococcus pneumonia, their occurrence in Cases 1, 3, 12, 19, and 20 cannot be attributed definitely to the effect of the sulfonamides.

CONCLUSIONS

These data indicate that hyperbilirubinemia and other evidence of hepatocellular damage may occur during the course of administration of sulfadiazine, as well as sulfathiazole, sulfapyridine, and sulfanilamide. Routine determina-

*From the Departments of Medicine and Physiology, Jefferson Medical College and the Laboratory of Biochemistry, Jefferson Hospital, Philadelphia.
†Ross V. Patterson Fellow in Gastroenterology.
Received for publication, July 13, 1942.

as outlined by Bliss and Marks.[6] The variance ascribable to differences in patients was about 25 per cent of the total. This points to the necessity of using a considerable number of patients and emphasizes the wisdom of having each patient serve as his own control. The "t" value of 6.305 ($P = < 0.001$) again shows that dextrins were significantly more effective in raising the blood sugar level than was dextrose.

It is impossible to state with certainty the reason for dextrin causing a higher and more prolonged rise in the blood sugar level than with dextrose itself. It is generally held that dextrins in the intestine undergo enzymic hydrolysis to maltose and then to dextrose, which is absorbed in the usual manner. The time factor of this hydrolysis may be the reason for the prolonged rise of blood dextrose after dextrin. Two other possibilities also present themselves: better absorption of the dextrose derived from dextrin, or a slower storage of the dextrose as glycogen by liver and muscle, although no evidence exists as to why such dextrose should behave different from pure dextrose. It is also recognized that the dextrins may on hydrolysis yield some slowly metabolized reducing substance which could react with the Shaffer-Somogyi reagents. No information is available on this point.

SUMMARY AND CONCLUSIONS

1. Twenty-three patients with chronic ulcerative colitis were studied as to the oral dextrose and oral dextrin tolerance tests.

2. One hour after dextrose administration the true blood dextrose had risen 42 per cent over the average fasting level while after the dextrin administration the blood dextrose had in the same time increased 70 per cent.

3. Two hours after dextrose the blood dextrose level was 7 per cent above the fasting level while the level in the dextrin group was 21 per cent above the fasting level.

4. Three hours after dextrose the blood dextrose level was 10 per cent below the fasting level while in the dextrin group it was 6 per cent above the fasting level.

5. A dextrin preparation maintained significantly higher blood dextrose levels for three hours than did dextrose in a group of patients with chronic ulcerative colitis. These findings have led to the assumption that in the patients studied dextrin was better absorbed from the intestine than was dextrose.

REFERENCES

1. Bercovitz, Z., and Page, R. C.: Metabolic and Vitamin Studies in Chronic Ulcerative Colitis. In press.
2. Groen, J.: The Absorption of Glucose From the Small Intestine in Deficiency Disease, New England J. Med. 218: 247, 1938.
3. Shaffer, P. A., and Somogyi, M.: Copper-Iodometric Reagents for Sugar Determination, J. Biol. Chem. 100: 695, 1933.
4. Fisher, R. A., and Yates, F.: Statistical Tables, Edinburgh and London, 1938, Oliver & Boyd.
5. Browne, C. A., and Zerban, F. W.: Physical and Chemical Methods of Sugar Analysis, ed. 3, New York, 1941, John Wiley & Sons, page 1006.
6. Bliss, C. E., and Marks, H. P.: The Biological Assay of Insulin. I. Some General Considerations Directed to Increasing the Precision of the Curve Relating Dosage and Graded Response, Quart. J. Pharm. & Pharmacol. 12: 82, 1939.

10	Gonococcus urethritis	Sulfathiazole	?	4 5 6	7.3 5.2 2.4	1.4 1.7 2.7	0.9 1.0 1.7	10 20	1:200 1:100 1:100	1:5	Albumin casts	80	4.2	6.3
11	Gonococcus urethritis	Sulfathiazole	?	5	9.2	2.5	1.2	10						
12	Pelvic peritonitis	Sulfathiazole	20	3	6.2	1.6	0.7							
13	Pelvic peritonitis	Sulfathiazole	20	3	5.6	6.6	2.5	15	1:50			72	4.0	10.6
14	Gonococcus urethritis	Sulfathiazole	10	2	8.0	1.0	0.5		1:400					
15	Gonococcus urethritis	Sulfapyridine	12	2 7 13	4.2 0.0 0.0	1.5 1.5 0.5	1.0 0.8	15 15 0	1:50 1:50 1:20	1.6 3.0	Blood crystals	90	4.6	7.9
16	Pyelonephritis	Sulfapyridine	40	7 8 12 35 39	9.1 6.8 0.5 0.0 0.0	2.0 5.4 5.9 1.8 1.0	0.8 3.0 3.0 1.0 0.7	40		1.6		(77) 68 51 89	(4.0) 3.7 2.8 4.6	(12.0) 10.3 18.0 13.2
17	Pyelonephritis	Sulfadiazine	12 18	2 3 6 8	6.2 9.3 2.0 0.5	2.1 3.2 1.8 0.8	1.4 2.0 1.3	20 30 10	1:100 1:100 1:50	1.1		(82) 80	(4.5) 3.9	(12.6) 9.2
18	Pyelonephritis	Sulfadiazine	18	3 4 7 12	7.1 7.0 5.3 0.6	1.7 2.2 2.1 1.2	1.3 1.6 1.6 1.0	30 30 20	1:200 1:100 1:50	1.3		(72) 68	(3.7) 3.5	(9.8) 8.4
19	Pneumococcus pneumonia	Sulfadiazine	18	3 4	9.6 6.8	1.5 1.3	0.6 0.5	0	1:200		Albumin casts	82	4.5	19.0
20	Pneumococcus pneumonia	Sulfadiazine	6 22	1 2 5 6 8	4.7 11.7 4.4 6.6	4.2 2.6 1.3 1.0	3.0 1.9 0.7 0.5	30	1:100	1.9		(85) 60 82	(3.9) 3.5 4.5	(25.0) 22.0 10.0

*Retention in blood thirty minutes after 2 mg. per kilogram.
†Excretion four hours after 6 Gm. of sodium benzoate by mouth.
‡Figures in parentheses values before sulfonamide therapy.

TABLE I

CASE	CONDITION	SULFONAMIDE	DOSE (GM.)	DAYS	BLOOD SULFONAMIDE (MG. PER 100 C.C.)	SERUM BILIRUBIN TOTAL (MG. PER 100 C.C.)	SERUM BILIRUBIN DIRECT (MG. PER 100 C.C.)	BROMSULFALEIN RETENTION* (%)	UROBILINOGEN IN URINE (URINE DILUTION)	HIPPURIC ACID EXCRETION† (GM.)	OTHER URINE FINDINGS	HB. %	ERYTHROCYTES (IN MILLIONS)	LEUCOCYTES (IN THOUSANDS)
1	Salpingitis Pelvic peritonitis	Sulfanilamide	8#	5	8.0	1.1	0.66					(78)	(4.2)	(14.6)‡
												55	3.8	15.2
			12	9	7.2	1.5	0.8		1:100			54	2.9	2.9
2	Cecostomy	Sulfanilamide	8#	2	6.5							(80)	(4.3)	(16.0)
				4	8.2	1.1	0.5					110	4.1	11.2
			32	8		3.8	2.5							
				9	7.8	5.0	3.1	30	1:800		Bilirubin	78	3.4	12.0
				10	2.8	5.0	3.1							
				14		3.0	1.7	10	1:20		Bilirubin	71	3.9	10.7
				28		0.4		0	1:20					
3	Pelvic abscess	Sulfanilamide	24	3	4.0	5.3	4.0					(80)	(4.0)	(8.2)
				8	0.0	10.5	7.3					75	4.0	8.9
4	Tonsillitis	Sulfanilamide	18	3	4.3	1.2	0.2		1:200					18.0
5	Acute appendicitis	Sulfanilamide	28	3	7.3	3.0	1.4		1:200		Blood, albumin casts	(82)	(4.2)	(16.9)
												84	4.2	10.0
6	Acute appendicitis	Sulfanilamide	16	2	4.4	3.7	0.7		1:50	1.1	Bilirubin	90	4.8	19.6
			24	5		2.5	1.0		1:100		Bilirubin	97	4.8	26.4
7	Gonococcus urethritis	Sulfanilamide	?	1	6.3	5.9	0.5					52	2.8	4.2
				3		4.6	0.5	0	1:300					
				4	2.1	2.9	0.7							
				5		1.6	0.8		1:500			58	3.0	5.7
				6		1.1	0.5							
				10	0.2	1.0	0.5							
				12	0.0	0.4	0.2		1:40			62	3.5	5.4
8	Acute appendicitis	Sulfathiazole	17.6	6	14.0	1.8	1.0					(65)	(3.5)	(25.6)
												60	3.8	19.7
9	Acute appendicitis	Sulfathiazole	10	2	8.6	2.0	1.2		1:400					

LABORATORY METHODS

GENERAL

A STUDY OF THE PHENOMENON OF ERYTHROCYTE SEDIMENTATION*

IV. Venous Stasis, Delay, Temperature of Sample, Containers, Agitation, and Quantity of Formed Elements as Sources of Technical Variations

Roy E. Nichols, B.V.Sc., M.Sc., Ph.D.,† Lafayette, Ind.

In PREVIOUS publications a critical survey of considerable literature,[1] choice of a reliable technique for the measurement of sedimentation,[2] and evidence substantiating my choice of the kind and quantity of anticoagulant[3] were presented. It is the purpose of this paper to discuss additional sources of technical variation and evidence of their control.

Free flow of blood without venous stasis cannot be accomplished in the horse unless a large enough needle is employed—at least a No. 10 gauge needle, or better yet, a fair-sized cannula. Repeated bleeding with a needle of this size creates considerable damage and is not advised. In lieu of the use of a large needle, a sample can be taken with a syringe (50 c.c. glass) and a smaller needle (No. 15 gauge 2 inch) in such a way that the effects of venous stasis can be avoided. Proof of this statement lies in the material which follows.

Five collecting vials, each containing the same kind and amount of anticoagulant, were filled to the 10 c.c. mark from the 15 gauge 2 inch needle inserted in the jugular vein. Venous pressure was continuous throughout the fillings and was accomplished by thumb pressure 5 to 6 inches below the needle and in degree was sufficient only to allow blood to run from the needle. Samples were allowed to settle in the vials at room temperature and then were shaken mechanically in a uniform manner. A Wintrobe tube was filled from each vial and placed in a rack to insure its perpendicularity. Results were:

Aliquot No.	M_s‡	t_{M_s}	$\angle^s M_s$
1	26 mm.	15-20 min.	30° 30′
2	20 mm.	{10-15 / 15-20} min.	37° 20′
3	18 mm.	10-15 min.	40° 20′
4	19 mm.	15-20 min.	39° 40′
5	24 mm.	15-20 min.	32° 20′

*From a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the Ohio State University, June, 1941.

†Comparative Physiologist, Department of Veterinary Science, Purdue University.

Received for publication, April 3, 1942.

‡*Definitions:*

M_s = Maximum five-minute descent in millimeters of the line of demarcation between cells and plasma.

t_{M_s} = Times in minutes between which M_s occurs. Since with the photographic apparatus one minute is the equivalent of 3 mm., this time-distance ratio is retained for the "field" technique.

$\angle M_s$ = Angle opposite the horizontal side of a right triangle having M_s as the perpendicular side and five minutes, or 15 mm., as the horizontal side.

tions of serum bilirubin concentration and tests of hepatic function would probably reveal a much higher incidence of hepatic functional impairment than has been reported heretofore following use of these agents.

REFERENCES

1. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. 119: 481, 1937.
2. Spink, W. W.: Sulfanilamide and Related Compounds in General Practice, Chicago, 1941, Year Book Publishers, Inc.
3. Long, P. H., Haviland, J. W., Edwards, L. B., and Bliss, E. A.: The Toxic Manifestations of Sulfanilamide and Its Derivatives, J. A. M. A. 115: 364, 1940.
4. Garvin, C. F.: Toxic Hepatitis Due to Sulfanilamide, J. A. M. A. 111: 2283, 1938.
5. Cline, E. W.: Acute Yellow Atrophy of the Liver Following Sulfanilamide Medication, J. A. M. A. 111: 2384, 1938.
6. Jackson, H. C., and Coller, F. A.: The Use of Sulfanilamide in the Peritoneum, Experimental and Clinical Observations, J. A. M. A. 118: 194, 1942.
7. Watson, C. J., and Spink, W. W.: Effect of Sulfanilamide and Sulfapyridine on Hemoglobin Metabolism, Arch. Int. Med. 65: 825, 1940.
8. Berger, S. S., and Applebaum, H. S.: Toxic Hepatitis Due to Sulfanilamide: Report of Fatal Case With Histopathologic Findings in Liver, J. Lab. & Clin. Med. 26: 785, 1941.
9. Bannick, E. F., Brown, A. E., and Foster, F. P.: Therapeutic Effectiveness and Toxicity of Sulfanilamide and Several Related Compounds, J. A. M. A. 111: 770, 1938.
10. Finland, M., Strauss, E., and Peterson, O. L.: Sulfadiazine: Therapeutic Evaluation and Toxic Effects on Four Hundred and Forty-Six Patients, J. A. M. A. 116: 2642, 1941.

Another example:

Aliquot No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%*
1	18 mm.	20-25 min.	40° 10′	39
2	18 mm.	15-20 min.	40° 10′	38.3
3	15 mm.	20-25 min.	45°	39½
4	18 mm.	20-25 min.	40° 10′	39½
5	18 mm.	15-20 min.	40° 10′	38½

This method is an improvement over continuous application of the thumb, but it still does not yield as comparable results as the technique with no stasis. In other words, if no thumb pressure is applied, except just enough to raise the vein to insert the needle, and if, after a moment's hesitation to allow free flow through the vein, a single sample is drawn into a clean dry glass syringe, and if five 10 c.c. aliquots are immediately supplied from the syringe, then results of determinations of M_5, t_{M_5}, and $\angle^s_{M_5}$ as also photographed curves of *sedimentation* from these aliquots will be comparable, for example:

Aliquot No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%
1	24 mm.	15-20 min.	32° 30′	34.5
2	23 mm.	15-20 min.	33° 30′	34.5
3	24 mm.	15-20 min.	32° 30′	35.0
4	25 mm.	15-20 min.	31° 20′	34.0
5	25 mm.	15-20 min.	31° 20′	34.5

This evidence, which may be repeated at will, is convincing enough to me that stasis of the vein at the time of withdrawal of a blood sample for the measurement of sedimentation should be avoided.

Delay.—Next let us consider the effects of delay in starting measurement following bleeding. Fig. 1, derived from three-paired samples, taken from one bleeding is typical of results. The oxalate mixture of Heller and Paul[4] was the anticoagulant used.

Ex. No.	Time after bleeding	em†	t_{em}	$\angle^s_g$
1	60 min.	28 mm.	12.8 min.	23° 00′
2	60 min.	28 mm.	11.8 min.	22° 30′
3	114 min.	30 mm.	11.8 min.	24° 40′
4	114 min.	29 mm.	11.0 min.	24° 10′
5	139 min.	20 mm.	10.3 min.	31° 30′
6	139 min.	21 min.	11.0 min.	31° 10′

Ex. No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%
1	28 mm.	10-15 min.	28° 30′	31
2	29 mm.	10-15 min.	27° 40′	32
3	28 mm.	10-15 min.	28° 30′	30.7
4	29 mm.	10-15 min.	27° 40′	31
5	19 mm.	10-15 min.	38° 50′	31
6	21 mm.	10-15 min.	36° 00′	30.7

*V% = Volume per cent of packed cells.

†em = The perpendicular distance from the estimated point of change between the vertical flexion and horizontal flexion of the photographically *recorded* sigmoid curve of sedimentation of a sample and the top line of the photograph representing 100 mm. of blood column height, i.e., estimated "fall" in millimeters at the point of change.

t_{em} = Time in minutes between the time of filling of the Wintrobe tube and the time that the point of change between the vertical flexion and horizontal flexion of the *photographically* recorded sigmoid curve of sedimentation of a sample occurs (one minute = 3 mm.).

$\angle_g$ = The least angle between the steepest slope of the sigmoid curve of sedimentation of a sample and perpendicularity.

Aliquot No.	M_5	t_{M_5}	$\angle^{s}{M_5}$
1	19 mm.	10-15 min.	39° 40′
2	21 mm.	15-20 min.	36° 00′
3	15 mm.	20-25 min.	45° 00′
4	16 mm.	10-15 min.	44° 30′
5	17 mm.	15-20 min.	41° 20′

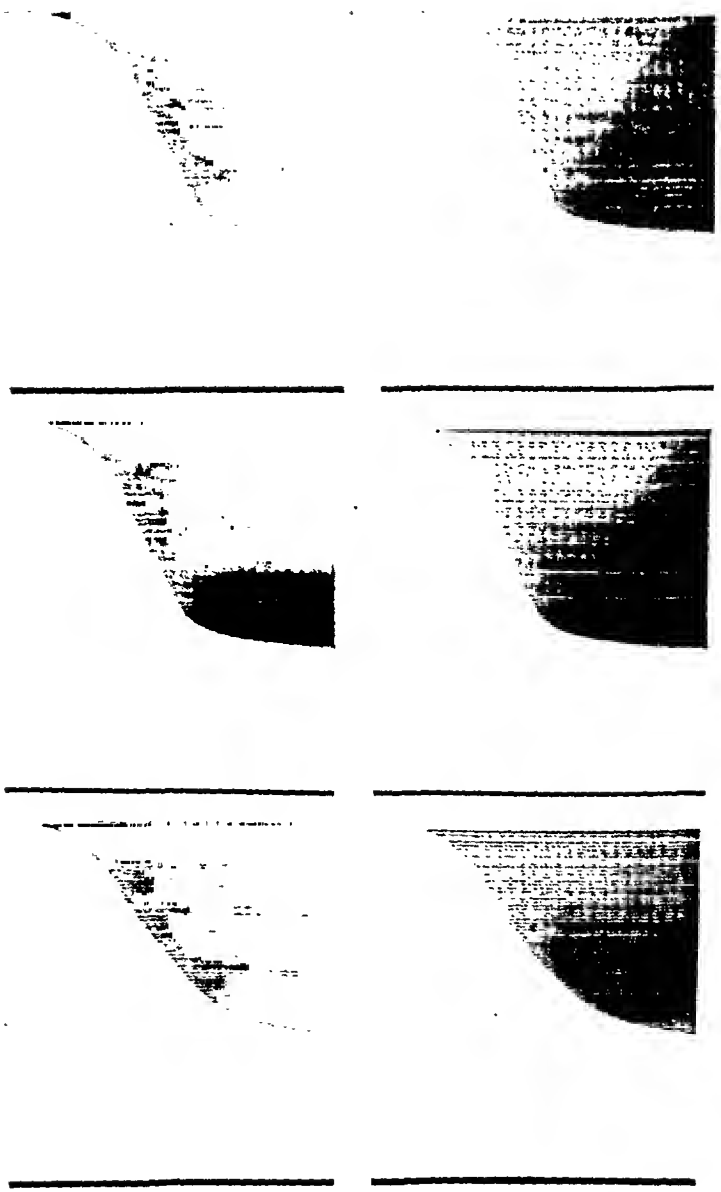


Fig. 1.

To show what happens when the thumb is removed after filling each collecting vial and is reapplied before filling the next:

Aliquot No.	M_5	t_{M_5}	$\angle^{s}{M_5}$
1	22 mm.	15-20 min.	34° 40′
2	23 mm.	15-20 min.	33° 30′
3	26 mm.	10-15 min.	30° 20′
4	22 mm.	10-15 min.	34° 40′
5	23 mm.	10-15 min.	33° 30′

With the temperatures tested the sedimentation rate increases as temperature rises. As yet no evidence has appeared to convince me that body temperature has any advantage over room temperature, or vice versa. No attempt has been made to demonstrate any mathematical relationship between temperature and sedimentation, although the possibility of such may exist.

Containers.—The reproducibility of the following tubes was tested:

Blood column—100 × 3 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$	V%
1	18 mm.	20-25 min.	40° 20′	39.3
2	18 mm.	{15-20 / 20-25} min.	40° 20′	39.3
3	18 mm.	15-20 min.	40° 20′	39.5
4	17 mm.	15-20 min.	42° 00′	39
5	18 mm.	15-20 min.	40° 20′	39

Blood column—200 × 3 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	29 mm.	15-20 min.	27° 40′
2	30 mm.	15-20 min.	27° 00′
3	28 mm.	10-15 min.	28° 30′
4	28 mm.	10-15 min.	28° 30′
5	27 mm.	{10-15 / 15-20} min.	29° 20′

Blood column—100 × 6 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	16 mm.	20-25 min.	43° 40′
2	16 mm.	25-30 min.	43° 40′
3	17 mm.	25-30 min.	42° 00′
4	16 mm.	20-25 min.	43° 40′
5	16 mm.	20-25 min.	43° 40′

Blood column—200 × 2 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	6 mm.	30-35 min.	67° 10′
2	18 mm.	{25-30 / 30-35} min.	40° 20′
3	19 mm.	{20-25 / 25-30 / 30-35} min.	38° 50′
4	19 mm.	25-30 min.	38° 50′
5	14 mm.	30-35 min.	46° 10′

Another example of 200 × 2 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	23 mm.	{10-15 / 15-20} min.	33° 40′
2	15.5 mm.	40-45 min.	44° 30′
3	24 mm.	20-25 min.	32° 30′
4	23 mm.	20-25 min.	33° 40′
5	21 mm.	20-25 min.	36° 00′

As can be seen all have fair reproducibility except the 200 × 2 mm. columns. Even with many efforts to fill uniformly these tubes, erratic results ensued. Despite these efforts I still feel that it is more reasonable to believe that variations are in a large part the result of discrepancies in tube filling and not of capillarity in a 2 mm. tube. Horse blood will not flow from an

The following is an example where potassium oxalate alone is used as an anticoagulant (Fig. 2). Approximately a ninety-minute interval exists between Example 1 and Example 2, both of which are from the same sample of blood.

Ex. No.	em	t_{em}	$\angle^s_g$
1	27 mm.	11.0 min.	26° 50′
2	8 mm.	9.5 min.	69° 20′

Ex. No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%
1	29 mm.	5-10 min.	27° 40′	40.7
2	8 mm.	5-10 min.	71° 40′	39.7

There appears to be a slowing of the rate of separation as time progresses. Examination of many samples suggests that with the ammonium and potassium oxalate mixture of Heller and Paul there may be a slight increase in rate preceding the slowing effect. However, tests run within approximately two hours yield fairly comparable results with this anticoagulant mixture. With some anticoagulants, particularly potassium oxalate, the effects of delay are apparently more pronounced and seemingly the more anticoagulant used the sooner slowing takes place.

Fig. 2.

Temperature.—Multiple determinations of rates at almost any single temperature are repeatable, but in the interest of standardization one should obviously not attempt comparison of samples at widely different temperatures. For example:

Aliquot No.	Temperature	M_5	t_{M_5}	$\angle^s_{M_5}$	V%
1	38° C.	29 mm.	10-15 min.	27° 30′	36.5
2	38° C.	30 mm.	10-15 min.	27° 00′	36.5
3	26° C.	22 mm.	10-15 min.	34° 40′	37
4	26° C.	22 mm.	15-20 min.	34° 40′	36.7
5	6° C.	3 mm.	85-100 min.	78° 20′	37 +
6	6° C.	2.5 mm.	75-80 min.	80° 10′	37.3

With the temperatures tested the sedimentation rate increases as temperature rises. As yet no evidence has appeared to convince me that body temperature has any advantage over room temperature, or vice versa. No attempt has been made to demonstrate any mathematical relationship between temperature and sedimentation, although the possibility of such may exist.

Containers.—The reproducibility of the following tubes was tested:

Blood column—100 × 3 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$	V%
1	18 mm.	20-25 min.	40° 20′	39.3
2	18 mm.	{15-20 / 20-25} min.	40° 20′	39.3
3	18 mm.	15-20 min.	40° 20′	39.5
4	17 mm.	15-20 min.	42° 00′	39
5	18 mm.	15-20 min.	40° 20′	39

Blood column—200 × 3 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	29 mm.	15-20 min.	27° 40′
2	30 mm.	15-20 min.	27° 00′
3	28 mm.	10-15 min.	28° 30′
4	28 mm.	10-15 min.	28° 30′
5	27 mm.	{10-15 / 15-20} min.	29° 20′

Blood column—100 × 6 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	16 mm.	20-25 min.	43° 40′
2	16 mm.	25-30 min.	43° 40′
3	17 mm.	25-30 min.	42° 00′
4	16 mm.	20-25 min.	43° 40′
5	16 mm.	20-25 min.	43° 40′

Blood column—200 × 2 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	6 mm.	30-35 min.	67° 10′
2	18 mm.	{25-30 / 30-35} min.	40° 20′
3	19 mm.	{20-25 / 25-30 / 30-35} min.	38° 50′
4	19 mm.	25-30 min.	38° 50′
5	14 mm.	30-35 min.	46° 10′

Another example of 200 × 2 mm.:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$
1	23 mm.	{10-15 / 15-20} min.	33° 40′
2	15.5 mm.	40-45 min.	44° 30′
3	24 mm.	20-25 min.	32° 30′
4	23 mm.	20-25 min.	33° 40′
5	21 mm.	20-25 min.	36° 00′

As can be seen all have fair reproducibility except the 200 × 2 mm. columns. Even with many efforts to fill uniformly these tubes, erratic results ensued. Despite these efforts I still feel that it is more reasonable to believe that variations are in a large part the result of discrepancies in tube filling and not of capillarity in a 2 mm. tube. Horse blood will not flow from an

inverted 3 mm. tube and seldom from a 6 mm. tube, yet with these bores replication is possible. Why the difference? Of course, with the 200 mm. tubes longer needles had to be employed for filling. Since these needles are No. 16 gauge and their outer diameter is 1½ mm., they nearly occlude the 2 mm. bore. In the necessary manipulation of the filling of the tube with a needle of this size, in all probability disturbance to the blood is greater in some fillings than in others, resulting in more complete breaking up of clumps of cells. Since the same needles can be used for filling larger bore tubes and replicate results ensue, it is thought that they are not in fault. Smaller bore needles, though they too offer disadvantages from an agitation standpoint, will have to be tried.

Next a comparison of 100 × 3 mm. and 200 × 3 mm. columns was made on aliquots of the same sample of blood. All tubes were filled with No. 16 gauge 11 inch needles:

Blood column—100 × 3 mm.:

Aliquot No.	M_5	$^{t}M_5$	$\angle^{s}M_5$
1	16 mm.	20-25 min.	43° 40′
2	15 mm.	20-25 min.	45° 00′
3	17 mm.	25-30 min.	42° 00′

Blood column—200 × 3 mm.:

Aliquot No.	M_5	$^{t}M_5$	$\angle^{s}M_5$
4	20 mm.	20-25 25-30 min.	37° 30′
5	22 mm.	20-25 min.	34° 50′
6	21 mm.	20-25 min.	36° 10′

Four 200 × 3 mm. tubes were filled to different levels:

Aliquot No.	Column height	M_5	$^{t}M_5$	$\angle^{s}M_5$	$\%M_5$*	$\angle^{s}\%M_5$
1	200 mm.	22.5 mm.	20-25 min.	34° 00′	11.25 mm.	52° 20′
2	150 mm.	18.5 mm.	10-15 min.	39° 30′	12.3 mm.	49° 50′
3	100 mm.	24 mm.	10-15 min.	32° 30′	24 mm.	32° 30′
4	50 mm.	13 mm.	5-10 min.	48° 30′	26 mm.	30° 20′

As can be seen by examination of these trends the actual maximum five-minute drop in the line of demarcation between cells and plasma is greater in 200 mm. columns than in 100 mm. Results in tubes of like bore filled to four different levels suggest that M_5 may be less and less as the height of the column is decreased.

If, however, one were to reduce the actually obtained values of M_5 to a percentage basis, then values of $\%M_5$ are not equal but increase as the column height decreases—additional indication that there is not a simple arithmetical relationship between column height and maximum drop. Attempts to demonstrate some other type of mathematical relationship will have to be left for future work—such a relationship theoretically might exist.

For an example of comparison of like columns of different diameter:

Blood column—100 × 3 mm.:

Aliquot No.	M_5	$^{t}M_5$	$\angle^{s}M_5$
1	15 mm.	25-30 min.	45° 00′
2	15 mm.	20-25 min.	45° 00′
3	17 mm.	20-25 min.	42° 00′

*% M_5 equals M_5 reduced to a percentage basis.

Blood column—100 × 6 mm.:

4	16.5 mm.	20-25 min.	42° 50′
5	16.5 mm.	20-25 min.	42° 50′
6	17 mm.	20-25 min.	42° 00′

Results are close.

As examples of the effects of deviations from perpendicularity:

Aliquot No.	Angles	M_5	$^tM_5$	$\angle^s M_5$	V%
1	90°	24 mm.	10-15 min.	32° 30′	34.7
2	80°	31 mm.	5-10 min.	26°	34.3
1	90°	21 mm.	15-20 min.	36° 10′	38.00′
2	80°	28 mm.	10-15 min.	28° 30′	38.00′

Five-minute readings for the angulated tubes were read directly from the graduations of the tubes. Little calculation is necessary to show that despite this, values of M_5 are greater than those of the corresponding perpendicular tubes.

To summarize, longer columns yield greater actual values of M_5. M_5 is not directly proportional to column height, for when values of M_5 are reduced to percentage drops, these are greater in shorter columns. There is apparently no significant difference between columns of 3 mm. diameter and columns of 6 mm. diameter. Two millimeter columns should be avoided. Small deviations from perpendicularity are apparently sources of discrepancy.

Shaking.—As one may have noticed in the review of literature on agitation prior to starting measurement of sedimentation,[1] too little stress has been placed upon the necessity of uniform dispersion of elements before measurement is begun. It was first reasoned that, if multiple aliquots from the same sample of blood were shaken to a point where they would yield fairly close values of volume per cent, their sedimentation rates should agree. The fallacy of such reasoning was soon clearly demonstrated. Such reasoning would apply to a simple sedimenting system where the dispersed elements remain discrete, but not to blood in which the phenomena of rouleaux formation and agglutination take place, and more especially not to blood in which this aggregating tendency is very pronounced, as is the case with horse blood. It has been reported that no aggregation occurs intravascularly in healthy human beings but such has not been reported in healthy horses. In fact, Fahraeus[5] states that close examination of the finer vessels of the mesentery of the horse reveals that the corpuscles are so strongly aggregated that they do not separate during flowing. No suspension exists. The erythrocytes flow as a compact stream in the middle of the vessel, and only when the stream is slowed by pressure on the supplying arteries does this string show granulated outlines and finally break to pieces. Whether Fahraeus' observation can be classed as evidence that a certain degree of aggregation occurs intravascularly in the healthy horse is certainly debatable. The very removal of mesentery from the abdominal cavity for examination may have in itself caused a certain degree of slowing in the vessels, this favoring rouleaux formation. More especially might this be true since the aggregating tendency is naturally very great in horse blood.

The problem of obtaining a uniform suspension of elements, whether singular, rouleaux, or aggregates of rouleaux, has been indeed a difficult one. Naturally, the first method attempted consisted of filling tubes as soon as possible

after bleeding, the aliquots being agitated by hand until the samples for filling the Wintrobe tubes were drawn. Such yielded erratic results.

The next method tried consisted of allowing the collecting vials to set until separation was fairly complete, usually ten to twenty-five minutes, and then shaking them as uniformly as possible by hand. This method was not dependable.

Aliquot No.	M_s	${}^tM_s$	$\angle^s M_s$	V%
1	21 mm.	15-20 min.	36° 00′	35−
2	24 mm.	15-20 min.	32° 30′	35+
3	23.5 mm.	15-20 min.	33° 00′	35

Striking the collecting vial upon a rigid surface only created more incomparable results:

Aliquot No.	M_s	${}^tM_s$	$\angle^s M_s$	V%
1	20.5 mm.	10-15 min.	36° 40′	35
2	21 mm.	5-10 min.	36° 00′	35+
3	15 mm.	10-15 min.	45° 00′	35

In face of erratic results with manual agitation, mechanical means were attempted. Several styles of mechanical agitators were constructed and tried. Finally, one was built[2] with a vial holder suspended from four 7¾ inch spring steel arms. This was driven in such a way as to yield a ¾ inch path of motion over an arc of 7¾ inch radius, yet, since four arms were used, the vial remained in a perpendicular position. The fact that the ends of the stroke were slightly higher than the center of the arc is thought to be an added assistance in overcoming the effects of the force of gravity (which theoretically is ever present and must be counteracted before dispersion can take place). When the path of the arc was more shallow (12 inch radius), layering effects were frequently encountered. At present the speed is 564 strokes per minute. It is felt that since fairly comparable results can usually be obtained when tubes are filled from different heights of replicate aliquots of the same sample of blood, in all probability this agitator is at least practically sufficient.

Aliquot No.	Height	M_s	${}^tM_s$	$\angle^s M_s$	V%
1	¾	19 mm.	20-25 min.	38° 50′	41.7
2	½	18 mm.	20-25 min.	40° 20′	42
3	¼	17 mm.	20-25 min.	42° 00′	41.7
4	0	19 mm.	20-25 min.	38° 50′	42

Additional evidence of its efficiency is noted when five Wintrobe tubes are filled from the bottom of the same 10 c.c. sample:

Tube No.	Height	M_s	${}^tM_s$	$\angle^s M_s$	V%
1	0	16 mm.	20-25 min.	43° 40′	38
2	0	16 mm.	20-25 min.	43° 40′	38
3	0	16 mm.	15-20 min.	43° 40′	38.3
4	0	17 mm.	15-20 min.	42° 00′	38+
5	0	16 mm.	15-20 min.	43° 40′	38+

The foregoing examples were from samples which were allowed to settle; each sample was shaken for fifteen minutes by placing the collecting vials into the shaker one minute apart and filling the tubes one minute apart.

The first attempts at arriving at a safe time of mechanical agitation for samples immediately placed in the shaker yielded results shown in Fig. 3. Data from these examples:

Ex. No.	Time shook	em	t_{em}	$\angle^s_g$
1	2 min.	31 mm.	10.5 min.	22° 10′
2	3 min.	29 mm.	9.3 min.	20° 00′
3	3.5 min.	29 mm.	8.3 min.	17° 50′
4	4.5 min.	26 mm.	7 min.	21° 30′
5	5.5 min.	23 mm.	7 min.	17° 30′

Ex. No.	M_s	t_{M_s}	$\angle^s M_s$	V%
1	27 mm.	5-10 min.	29° 20′	31.8
2	29 mm.	5-10 min.	27° 20′	31.8
3	33 mm.	5-10 min.	24° 40′	31
4	35 mm.	5-10 min.	23° 20′	31
5	39 mm.	5-10 min.	21° 10′	31.3

As can be seen, five and one-half minutes at a speed of about 600 strokes per minute was apparently not sufficient to reduce aggregated elements to at least minimum size.

To determine whether results might be comparable if samples were drawn, immediately placed in the shaker, and agitated for fifteen minutes:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$	V%
1	17 mm.	25-30 min.	42° 00′	44
2	16 mm.	20-25 min.	43° 40′	43.5
3	13 mm.	20-25 min.	48° 20′	44
4	20 mm.	25-30 min.	37° 20′	44
5	20 mm.	25-30 min.	37° 20′	43.5

Results were still too variable.

If samples were immediately placed in the shaker and agitated for forty minutes:

Aliquot No.	M_s	t_{M_s}	$\angle^s M_s$	V%
1	23.5 mm.	10-15 min.	33° 00′	37−
2	23.5 mm.	10-15 min.	33° 00′	36.3
3	22 mm.	15-20 min.	34° 40′	36.7
4	23.5 mm.	10-15 min.	33° 00′	36.5
5	23.5 mm.	10-15 min.	33° 00′	36+

Results agree very well.

Apparently a certain amount of aggregation takes place even during shaking, and apparently a certain amount of time following bleeding must pass before agreeable replicate values can be obtained. From a practical standpoint, if equally as good results can be obtained by allowing the blood to separate before agitation as by prolonged agitation, the logical choice is to allow settling to take place first. There is additional experience in favor of this choice. In samples of very slow blood, agitation, if commenced immediately after bleeding, must be continued longer than in samples from fast blood. In slow blood one is always reasonably doubtful about reaching that phase of agitation where replication is possible. Where slow blood is allowed to settle first, in no case so far has there been evidence to suggest that agitation for fifteen minutes was insufficient.

It was first thought that if blood was allowed to separate first, speed alone might bring about uniformity of suspension. Blood was allowed to settle and

then was placed in the agitator between 500 and 600 strokes per minute for varying times. There was no visible hemolysis at this speed while at faster speeds there was occasional hemolysis in some pathologic cases.

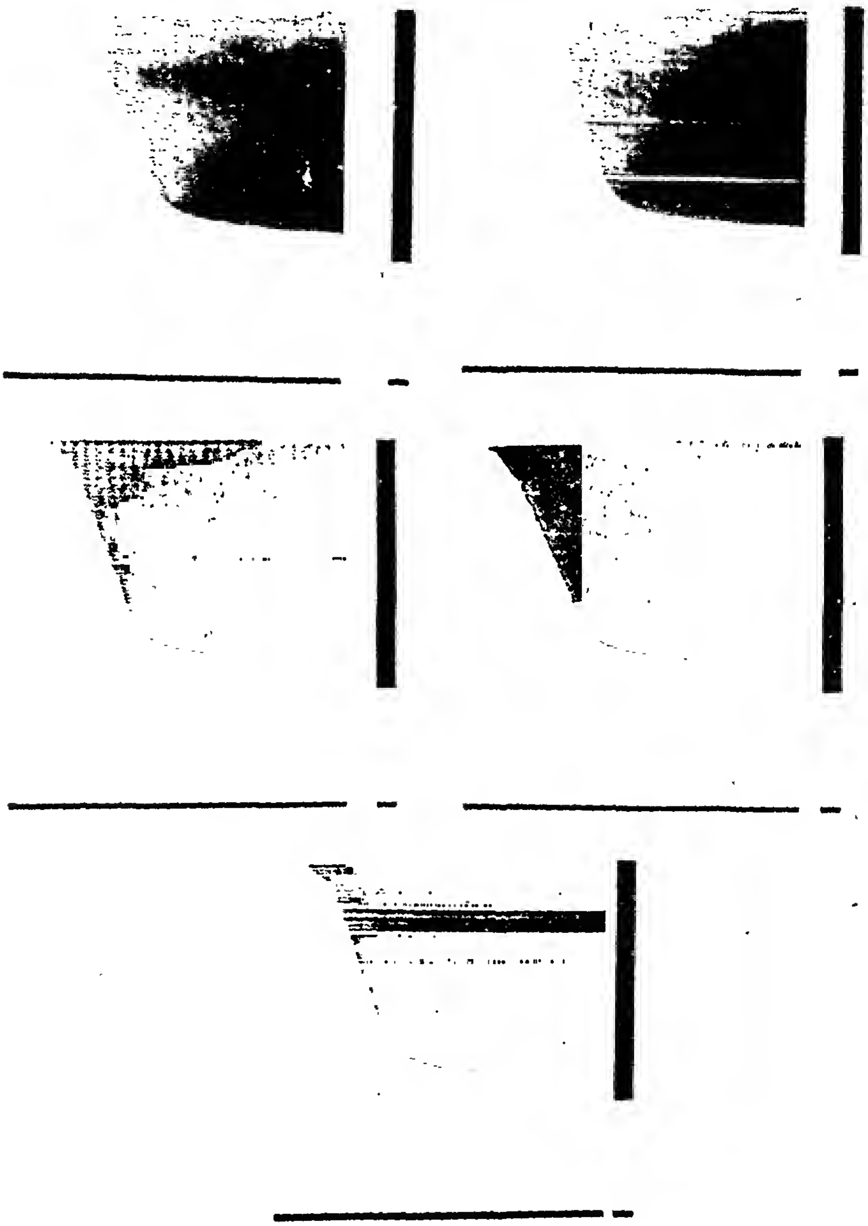


Fig. 3.

Results were similar to:

Aliquot No.	Time Shaken	M_5	${}^{t}M_5$	$\angle^{s}M_5$	V%
1	1 min.	12 mm.	15-20 min.	50° 30′	42½
2	5 min.	16 mm.	30-35 min.	43° 40′	43–
3	10 min.	18 mm.	20-25 min.	42° 40′	42–
4	15 min.	19 mm.	20-25 min.	38° 40′	42½

Shaking for thirty minutes after the samples have been allowed to separate yields no more comparable figures than shaking for fifteen minutes. In more rapid samples shaking for five minutes will yield comparable values, but I have greater confidence in fifteen minutes as a routine shaking time.

It was earlier mentioned[2] that variations in t_{em} and the limits of t_{M_5} were encountered even when values of em and M_5 were not far off. Volume percentages were within narrow limits, t_o-t_o* was carefully established, and all calculations and measurements were checked. At first it was thought that the mechanical agitation was at fault. Since at no time has it been possible to demonstrate microscopically that settled blood can be separated by agitation to a state of single cells—because rouleaux formation is so rapid in the equine species that shed blood no matter how quickly examined after bleeding cannot be found without agglutination—no definite proof of the efficacy of any type of agitation has been possible as yet. Means other than the microscope will have to be used.

However, since with some methods of filling the measuring tubes very close results were obtained while with others results were not so close, it was reasoned that in all probability the mechanical agitation merely reduced the solid elements to a certain degree of dispersion but not to the discrete state and that methods of filling measuring tubes were at fault. The early practice of removing the collecting vial from the shaker before withdrawal of blood for filling the measuring tube was stopped, and in its place was substituted the practice of withdrawing blood from the vial while the shaker was in motion, this being possible because of the short stroke of the apparatus. In addition to this, syringes attached to blunt No. 18 gauge 4½ inch needles were used in place of the earlier used glass pipettes furnished with the Wintrobe tubes, the latter being very subject to breakage when inserted into the moving vial. Results were no better.

Next it was reasoned that the suction of drawing the blood into a syringe (used earlier in the course of this problem) and the pressure necessary for its expulsion into a tube were probably in reality means of agitation in themselves; and if no uniformity existed in this practice, they might be responsible for some of the discrepancies observed. It was further reasoned that if this source of possible variation could be minimized, closer results would ensue. Since the hubs of these long-filling needles were round and fairly large, the rubber bulbs which are furnished with the Wintrobe filling pipettes were attached and tried. Before trial these bulbs were selected as to volume and degree of firmness, and only those which were comparable were employed. By using this equipment and placing more stress on forming a uniform "habit" of filling tubes, closer results of em, t_{em}, M_5 and t_{M_5} were obtained.

The problem of t_{M_5} has, however, not yet been completely solved, but a little reasoning reveals that since limits of t_{M_5} never pass beyond the next five-minute brackets and since, even if the limits of t_{M_5} are within different brackets, in practice em, t_{em}, $\angle^s{}_g$, M_5 and $\angle^s{}_{M_5}$ may be very close, this discrepancy is not too serious. After all t_{M_5} merely is a wide range within which t_{em} lies. Usually

*Time of filling the tube.

if t_{em} is close to one or the other of the limits of t_{M_5} in results where there is variation in t_{M_5}, it will be in the direction of t_{em}. Also where two readings of M_5 are close, t_{em} usually lies nearer the middle of these five-minute limits. In cases where M_5 lies between both five-minute limits, as it frequently does, t_{em} in most cases lies closer to the center five minute time. Since in practice t_{em} is usually not affected more than one minute, and rarely two minutes or more, and since in all probability $\angle^{s}{}_{g}$ in the photographic technique and $\angle^{s}{}_{M_5}$ in the "field" technique are better estimates of the speed of settling than values of either I_{em}* or I_{M_5}†, this deviation in t_{M_5} is thought not serious. Also since, if t_{em} happens to be close to one or the other of the limits of t_{M_5}, small variations in t_{em} may be sufficient to change the limits of t_{M_5}, in replicate sampling variations in values of t_{M_5}, apparently due to discrepancies in filling the measuring tubes, are not considered of distracting value.

Since replicate results agree very closely with the previously described technique of agitation and tube filling, this method is thought to be fairly dependable.

Volume Per Cent and Sedimentation.—Now comes a phase of this work which is indeed interesting and which I feel cannot be answered in a thesis purely on technique, but it might be in a problem of "apparently normal ranges." What is the relationship between the quantity of formed material and the rate at which this material separates out? Since it is quite universally recognized that the cells do not settle discretely, what effect does the quantity of formed elements have upon rouleaux formation and agglutination?

Rouleaux formation has been said to be merely a type of agglutination in which the cells, because of their specific shape, take on a nummular appearance, other physical factors apparently being similar. Is such unreasonable? Rouleaux, as observed microscopically, are said to attain a certain average length and from thence on agglutination is of rouleaux and not single cells. It has even been suggested[5] that agglutination continues until the whole mass of cells seems to form a spongelike architecture.

It has also been suggested by Balachowsky[6] that:

1. Red blood cells forming rouleaux and conglomerates constitute a network which is the skeleton of the blood.

2. Under the influences of weight and through a series of local depressions there occurs a general heaping of the blood skeleton on the bottom of the receptacle.

3. This blood skeleton is not deprived of solidarity for (a) the configuration of a given portion may remain unchanged for a long period of time. (b) The free upper border of the bulk of the erythrocytes preserves its shape for a long period. (c) There exists a force of resistance against the collapse of the blood skeleton other than viscosity.

4. The solidarity of the blood skeleton, being a function of the tonus of its constituents, is a new factor denominated "hematonia."

---

*I_{em} = An index $\frac{cm}{t_{em} \times 3}$.

†I_{M_5} = An index calculated by dividing the "falls" in millimeters between which M_5 occurs by their corresponding times $\times$ 3 (3 mm. = one minute).

Balachowsky feels that sedimentation of erythrocytes is not, as has been assumed, merely a simple phenomenon of sedimentation, but a more complicated reaction in which the effects of heaping play an important part. For this reason, he would dominate the phenomenon as the sedicompilation of erythrocytes.

If one closely watches a Wintrobe tube of horse blood, he can observe that the edges of the column of blood present a certain, ragged appearance, sometimes even before much settling has taken place. Is it unreasonable to assume that this is part of the agglutination phenomenon and that this effect may be general throughout the column? Another common place that this may be observed is in the pipette of a Dare hemoglobinometer. The layer of blood is sufficiently thin for one to watch the gradual agglomeration and settling.

Of course, one can observe a certain limitation to the size of rouleaux and clumps under a cover slip. The physical boundary of the blood column in one direction (height) is a matter of microns, and this in itself physically limits, to some extent at least, the degree of agglomeration. The dilution necessary to demonstrate rouleaux probably is in itself a limiting factor. However, cover-slip specimens of a sample of blood may show a certain limit to agglomerate size while tubes of the same sample may present evidence of mass agglomeration. In other words, in samples examined under a cover slip, mass agglomeration is quite free in two dimensions, but somewhat limited in the third, while in tubes, agglomeration is practically unlimited in three dimensions, at least before mass collapse.

Should it be said that the descent of the line of demarcation between cells and plasma represents a measure of size of agglutinates, or should it be said that the fall of this line of demarcation represents a measure of mass agglutination and mass collapse?

As to the effect of the quantity of formed material, cannot one say that, being one of the factors controlling the degree of agglutination as observed microscopically, it probably is one of the controlling factors of the degree of mass agglutination? Examinations of cover-slip preparations of various dilutions of cells and maximum five-minute falls on the same sample seem to substantiate this, as for example:

	V%	M_5	$^tM_5$	$\angle^s M_5$
Original sample:	43	21.9 mm.	10-15 min.	34° 40′
Volume altered to:	48	14 mm.	10-15 min.	46° 10′
"	39	25 mm.	5-10 min.	31° 30′
"	29.5	48 mm.	0-5 min.	17° 30′
"	20	61 mm.	0-5 min.	13° 50′
"	10.5	72 mm.	0-5 min.	11° 50′

Since the degree of agglutination was too great to count rouleaux and estimate the number of cells per rouleaux, the blood of the same animal was diluted (with 0.9 per cent sodium chloride) so that such might be accomplished. As expected, the size and number of agglomerates diminished with dilution, next the size and number of rouleaux diminished, until finally all single cells existed. Since the border of cells and plasma became more and more hazy the greater the dilution, actual estimations of M_5 were given up.

In dilutions showing hazy plasma, it will be noticed that that portion of the elements which does settle out rapidly gradually diminishes in volume as dilution increases, until finally the suspension comes apparently to its maximum stability. *This "titer" corresponds roughly at least to the point at* which only single cells are observed in the cover-slip preparations.

As purely a general observation there seems to be a concentration of elements in apparently healthy blood where the point of change in the sigmoid curve roughly numerically equals the percentage volume of elements, namely, at about 33⅓ per cent or ⅓, of the column height. The point of change in "normal" samples is seemingly less than 33⅓ mm. when the volume per cent is greater than 33⅓, and vice versa. Proof of this observation has not been attempted in this problem nor has any attempt been made to relate mathematically the point of change of curves of sedimentation with volume percentages because it is thought that such must entail examination of many "normal" samples and their adjustment to various concentrations—a task which would in itself entail more time and effort than can be afforded this thesis and could well be included in a study of normals. Nevertheless, Fahraeus[5] must have been thinking of a somewhat similar observation when he made this statement: "In general the sinking velocity of the upper corpuscle layer is diminished when it has sunk ⅓rd of the height of the blood column, i.e., in the tubes used by me (150 mms.) when the line of demarcation has descended 50 mms."

To theorize, if a mathematical relationship can be proved to exist between the point of change of the sigmoid curve representing the descent of the line of demarcation between cells and plasma and the percentage volume of formed elements, then with this knowledge, if the point of change is not as expected for a particular percentage, its deviation from the expected might reflect alterations produced by factors other than the volume percentage of elements. Such alterations might be estimated without standardization of the quantity of formed elements for the particular sample. Such no doubt would be an advantage.

To summarize in a general way, we seem to have least settling at dilutions where cells are discrete: a gradual increase in settling of at least a portion of the cells (probably as rouleaux or small clumps) as dilution becomes less; a point where maximum speed of separation seems to occur: then a gradual decrease in separation speed (as determined by the descent of the line of demarcation between cells and plasma) as concentration increases, there possibly being a mathematical relation between the slowing of the speed of separation and the increase in concentration.

REFERENCES

1. Nichols, R. E.: A Study of the Phenomenon of Erythrocyte Sedimentation. I. A Critical Survey of Literature, J. Lab. & Clin. Med. 27: 1317, 1942.
2. Nichols, R. E.: A Study of the Phenomenon of Erythrocyte Sedimentation. II. The *Establishment of a Reliable Measurement of the Phenomenon—Its Reproducibility* and Limitations, J. Lab. & Clin. Med. 27: 1410, 1942.
3. Nichols, R. E.: A Study of the Phenomenon of Erythrocyte Sedimentation. III. Anticoagulation as a Technical Source of Variations, J. Lab. & Clin. Med. 27: 1569, 1942.
4. Heller, V. A., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, J. Lab. & Clin. Med. 19: 777, 1934.
5. Fahraeus, Robin: The Suspension-Stability of the Blood, Acta med. Scandinav. 55: 1, 1921.
6. Balachowsky, S.: Hematonia: A New Factor Determining the So-Called Sedimentation Velocity of the Erythrocytes, Ann. de méd. 18: 201, 1925.

BLOOD CLOT RETRACTION*

I. Measurement of the Extracorpuscular Volume of the Clot

P. M. Aggeler, M.D., and S. P. Lucia, M.D., San Francisco, Calif., and
L. M. Hamlin, M.A., Berkeley, Calif.

Direct observation of the blood clot may reveal gross evidences of friability and deficient retraction. However, quantitative measurements are required to compare retraction of the blood clot of different patients, or of that of the same patient over a period of time. A method for estimating the degree of clot retraction, called the "serum volume index," was introduced by Boyce and McFetridge.[1] A quantity of blood, preferably 3 c.c., was collected in a graduated centrifuge tube and allowed to stand at room temperature. At the end of four hours the total volume of the specimen was noted, the clot was removed, and the residual volume of serum was recorded. In normal subjects the volume of serum was stated to be equivalent to 50 per cent of the volume of the specimen. There was a marked diminution of the serum volume in some jaundiced patients who suffered from abnormal bleeding. The authors expressed the deficiency of clot retraction in terms of the "serum volume index," which was obtained by dividing the volume of the serum by one-half of the total volume of the blood specimen. The index for normal subjects was 1.00, and values less than one were considered as indicative of a tendency to bleed. It was observed that if anemia were present, the serum volume would normally be greater than half the blood volume, and a correction based on the erythrocyte count was made in calculating the index.[2] It appears to us that in addition the mean volume of the red blood corpuscles and increases in the number of leucocytes may influence the results of the test. Nevertheless, the serum volume index is a valuable bedside test for the detection of deficient clot retraction.

Macfarlane[3] introduced a new method for measuring clot retraction which makes use of a graduated centrifuge tube into which is fitted a cork bored to receive a glass rod. The glass rod measures about 0.5 inch shorter than the tube and has a small expansion about 0.5 inch from the lower end. To perform the test, 5 c.c. of blood obtained by venipuncture are immediately introduced into the tube, the glass rod and cork are fitted, and the tube is placed in a water bath at 37° C. One hour after a firm clot has formed, the tube is removed from the bath, the clot is carefully freed and removed on the glass rod. The volume of serum is measured directly, and retraction is expressed in terms

*From the Departments of Medicine and Preventive Medicine, University of California Medical School, San Francisco, and the Department of Hygiene, University of California, Berkeley.

Aided by a grant from Mr. Frank Kennedy and from the Christine Breon Fund.
Received for publication, April 27, 1942.

of the percentage of the serum volume. The author found that the normal serum volume per cent fluctuated between 43.9 and 65.5 per cent. No correction was made for anemia.

The "clot retraction index" introduced by Mettier and Witts in 1939[3] is performed in a manner similar to that described by Macfarlane, except that a correction for anemia is made. The packed cell volume is added to the serum volume per cent and the sum is divided by two. In normal subjects the "clot retraction index" varies from 38 to 60. We have found the "clot retraction index" to be a useful clinical test.[6, 7]

In the course of a study of the various factors influencing the volume of the clot, we have found it convenient to express the character of the clot in terms of the extracorpuscular volume of the clot, which may be defined as the difference between the volume of the clot and the volume of the cells contained therein. It is our purpose to record the results of observations made in normal subjects and in patients suffering from abnormal bleeding.

METHOD

Slight modifications of Macfarlane's method for examining the clot were made. The upper end of a copper wire about 1 mm. in diameter is attached to a cork. The free end of the wire is bent in the shape of a hook. An arbitrary amount of venous blood, usually 5 c.c., is carefully transferred to a graduated centrifuge tube, and the cork and wire are fitted so that the hook is immersed in the upper layers of the specimen. After coagulation of the blood has occurred, the tube is placed in a water bath (37° C.) for one hour. It is then taken from the bath, the total volume of the specimen is recorded. the clot is elevated, allowed to drain and then removed. The residual volume of serum is measured, and when it is subtracted from the total volume of the specimen, the clot volume remains. To obtain the volume per cent of the blood clot, the observed volume of the clot is divided by the total volume of the specimen, and the quotient is multiplied by 100. (The measurement of the packed cell volume, including all cellular elements, is obtained by centrifugalization of a separate specimen of oxalated blood at 3,000 r.p.m. for thirty minutes.) The difference between the packed cell volume in per cent and the clot volume in per cent is the per cent of the total blood specimen occupied by the extracorpuscular volume of the clot. A value of zero implies that the clot is composed entirely of cells, and hence that excellent retraction has occurred. A high figure indicates that a large amount of extracorpuscular fluid is retained in the clot and that retraction is deficient. Occasionally, the clot volume is slightly smaller than the packed cell volume. This apparent discrepancy could be due to the inherent errors of the method, or it may be that fluid has been expressed from the cells, or that the spatial relationships of the cells are different when they are packed by centrifugal force in contrast to the agglomerations brought about by the natural retraction of the blood clot. Whatever the cause of this phenomenon, it always indicates excellent clot retraction.

Errors of the Method.—The inherent error of the method when tested by serial examination is approximately 5 per cent. The presence of the wire within the clot introduces a constant error of less than 0.5 per cent, which for con-

venience is neglected in the calculations. Occasionally, some of the cells may escape from the clot and settle at the bottom of the tube. This is frequently seen in specimens obtained from patients suffering from thrombocytopenia, polycythemia vera, and congenital hemolytic icterus. In these instances the serum is centrifugalized and the volume of the clot is corrected by adding to it the volume occupied by the escaped cells. The extracorpuscular volume of such clots will be less by an amount equal to the fluid which would be characteristically occluded in such clots had these cells remained within the clot. Such errors are slight and do not alter the trend of the results.

In all experiments the platelet counts were done on capillary blood after the method of Rees and Ecker, and the prothrombin concentration after the method of Quick.

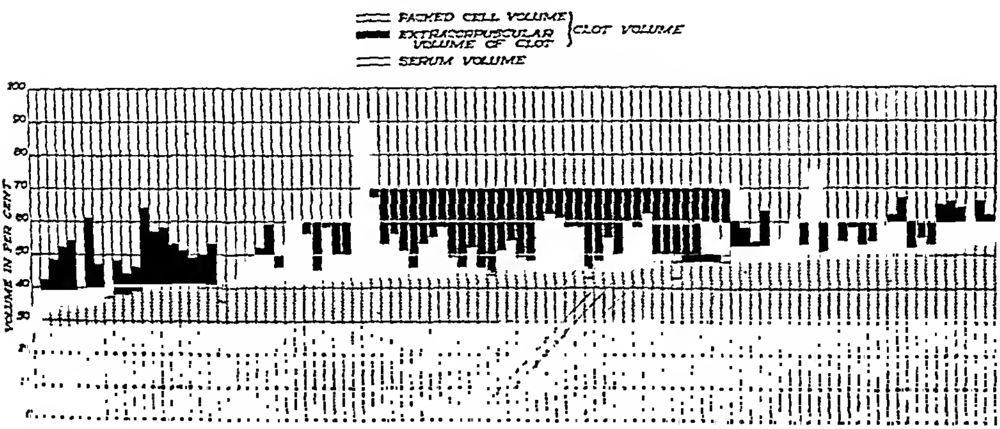


Fig. 1.—Relation between serum volume, packed cell volume, clot volume, and extracorpuscular volume of the clot in 100 normal subjects.

Material of the Study.—Specimens of blood from the following groups of persons were examined:

1. One hundred normal persons in whom the platelet counts were within normal limits (250,000 to 600,000) and in whom the prothrombin concentration was 70 per cent or greater.

2. Forty-two patients suffering from hypoprothrombinemia in whom the platelet counts were greater than 200,000 per cubic millimeter and in whom the prothrombin concentration was 50 per cent or less.

3. Thirty-five patients suffering from thrombocytopenia in whom the platelet count was 200,000 per cubic millimeter or less and in whom the prothrombin concentration was greater than 50 per cent.

RESULTS

The serum volume, clot volume, packed cell volume, and extracorpuscular clot volume expressed in per cent for each of 100 normal subjects are given in Fig. 1. The data for those with hypoprothrombinemia are given in Fig. 2, and for those with thrombocytopenia in Fig. 3. There did not appear to be any significant relationship between the packed cell volume and the extracorpuscular volume of the clot in any of these patients. In Fig. 4 the frequency distribution of the values for the extracorpuscular volume of the clot observed in each

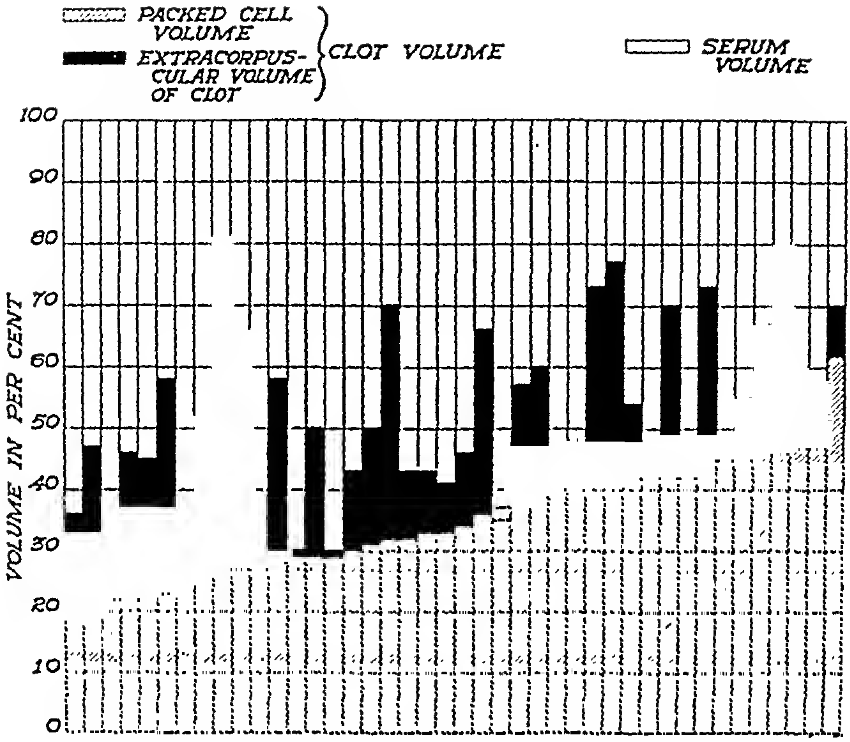


Fig. 2.—Relation between serum volume, packed cell volume, clot volume, and extracorpuscular volume of the clot in 42 subjects with hypoprothrombinemia.

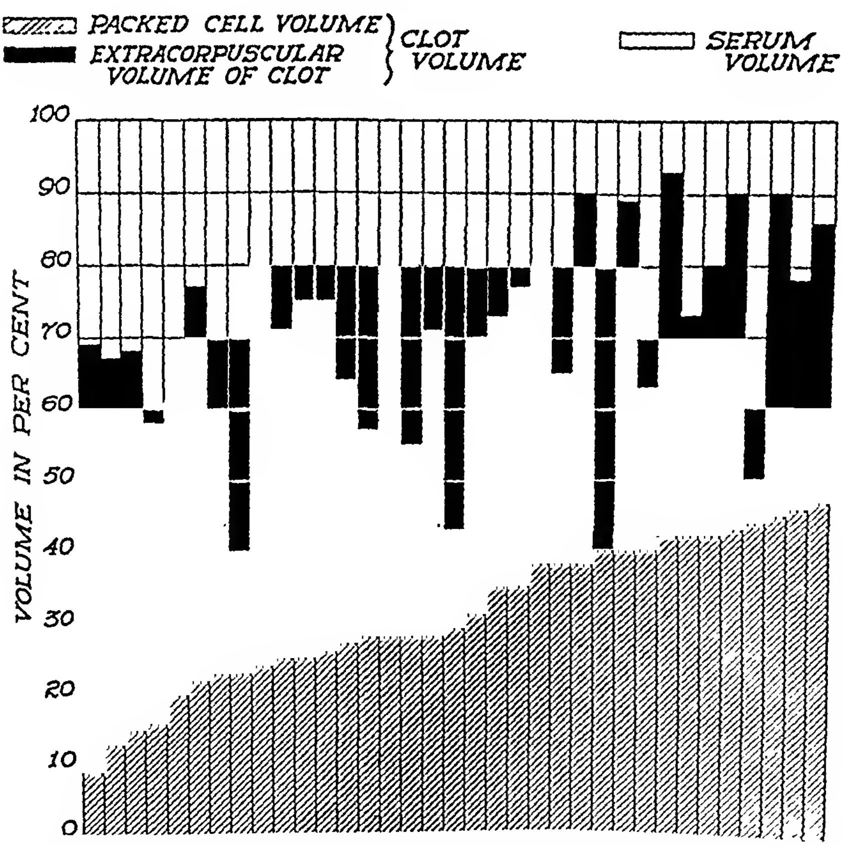


Fig. 3.—Relation between serum volume, packed cell volume, clot volume, and extracorpuscular volume of the clot in 35 subjects with thrombocytopenia.

of the three groups is given. It can readily be seen that the values for the extracorpuscular volume of the clot of many patients with hypoprothrombinemia overlap the normal range, while the values for most of the patients with thrombocytopenia fall outside the normal range. The results of the statistical analysis of these data are given in Table I. The standard statistical method was used in setting up the limits of normality. The limits of significance of the data were arbitrarily set at two standard deviations from the mean, which includes approximately 96 per cent of the observations. The mean is taken at the point of reference. All measures calculated were at least three times their sampling errors. The mean value of the extracorpuscular volume of the clot for the normal group was 9.1 per cent, the range of the values being − 5.9 to 24.1 per cent. The extracorpuscular volume of the clot was greater than 24.1 per cent in 13 of 42 patients with hypoprothrombinemia and in 30 of 35 patients with thrombocytopenia. The respective mean values for these groups were 18.2 and 38.9 per cent.

TABLE I

POINT AND RANGE MEASURES OF THE EXTRACORPUSCULAR VOLUME PER CENT OF THE BLOOD CLOT*

	MEAN	σ_x	M ± 1σ_x	M ± 2σ_x	MEDIAN (FROM OGIVE)
Normals (N=100)	9.1	7.5	1.6 − 16.6	(−) 5.9 − 24.1	8.7
Hypoprothrombinemia (N=42)	18.2	12.4	5.8 − 30.6	(−) 6.6 − 43.0	15.0
Thrombocytopenia (N=35)	38.9	14.1	24.8 − 53.0	10.7 − 67.1	42.3

*Using three sampling errors as a criterion, all measures are statistically significant.

A further study of the structure of normal and abnormal blood clots was made by histologic examination after fixation in 10 per cent formalin in absolute alcohol. In the normal clot (Fig. 5) the erythrocytes are densely packed. A poorly retracting clot from a patient suffering from thrombocytopenia is demonstrated in Fig. 6, which shows loose packing of the erythrocytes, uniform fragmentation of the clot, and irregular inclusion of large pools of serum.

The extracorpuscular volume of the clot is composed of fibrin and unexpressed serum. Experimentally, we have determined that the volume occupied by fibrin in the normal recalcified plasma is equal to approximately 1 per cent of the total specimen.

DISCUSSION

Macfarlane suggested a relationship between a decrease in packed cell volume and a reduction in the volume of the clot when retraction is normal. However, he states: "This relationship does not apply when, in abnormal cases, retraction is deficient. In such cases retraction may be brought to a standstill not by the volume of the cells contained within the clot, but by an inherent inability of the fibrin to contract to the extent normally attained. Under these conditions it is clear that a reduction in the cell volume cannot increase the extent of retraction.

"Thus, whereas it is possible to correct by a simple calculation for the effect of anemia if retractility is normal, no such correction can be applied if it is

deficient. Hence the application of a correction to pathological cases will produce distortion, since deficient retraction will be revealed in anemic cases more often than in those with a normal cell volume."

It appears to us that blood clot retraction may be controlled through an equilibrium existing between the retractile force of the fibrin on the one hand, and the opposing resistance to aggregation of the retained cells on the other. Their relative influence may vary considerably, depending upon many factors, including the volume of the packed cells and the quantity and quality of the platelets, prothrombin, and fibrin. In the blood of normal subjects conditions appear to be fairly well balanced so that the average extracorpuscular volume of the clot is approximately 10 per cent of the original specimen. In the abnormal subject the same forces operate, but the balance may be upset because the retractile power of the fibrin may be diminished, and equilibrium is established when the average extracorpuscular volume of the clot is relatively large.

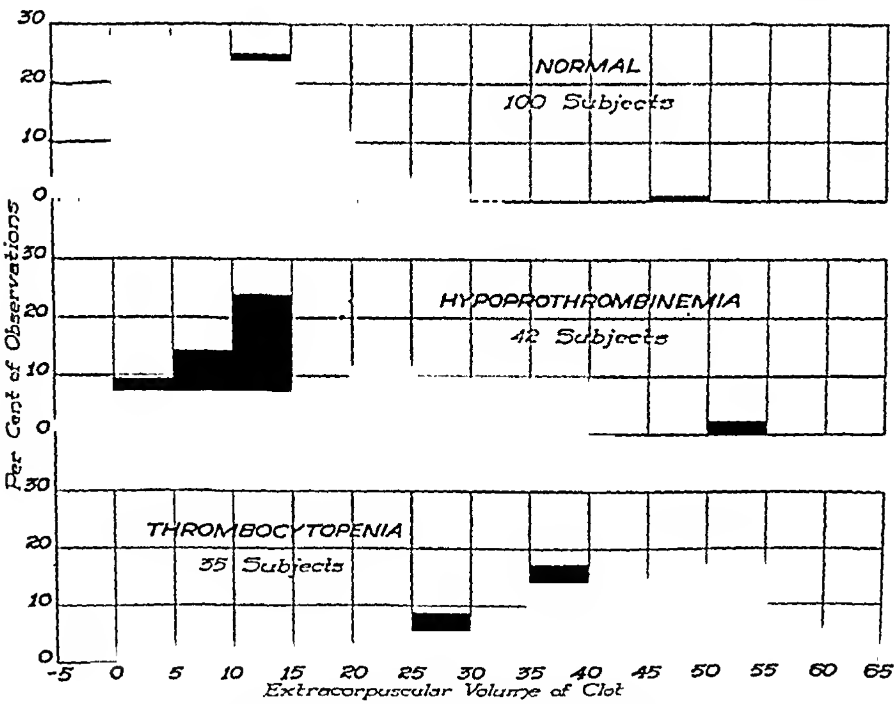


Fig. 4.—Frequency distribution of extracorpuscular volume of the blood clot in per cent of the total volume of the specimen.

When the packed cell volume is very low, and all other factors which influence clot retraction are normal, the maximum retractibility of fibrin may be achieved, and beyond this point there can be no further retraction regardless of the packed cell volume. It is obvious that this can happen only when clot retraction is excellent.

The volume occupied by the packed cells should be considered in estimating the degree of clot retraction. Failure to consider the effect of the cell volume on the volume of the clot may result not only in failure to detect deficient clot retraction in patients who have a normal cell volume, but also in the inability to recognize abnormal retraction in those who have severe anemia. Further-

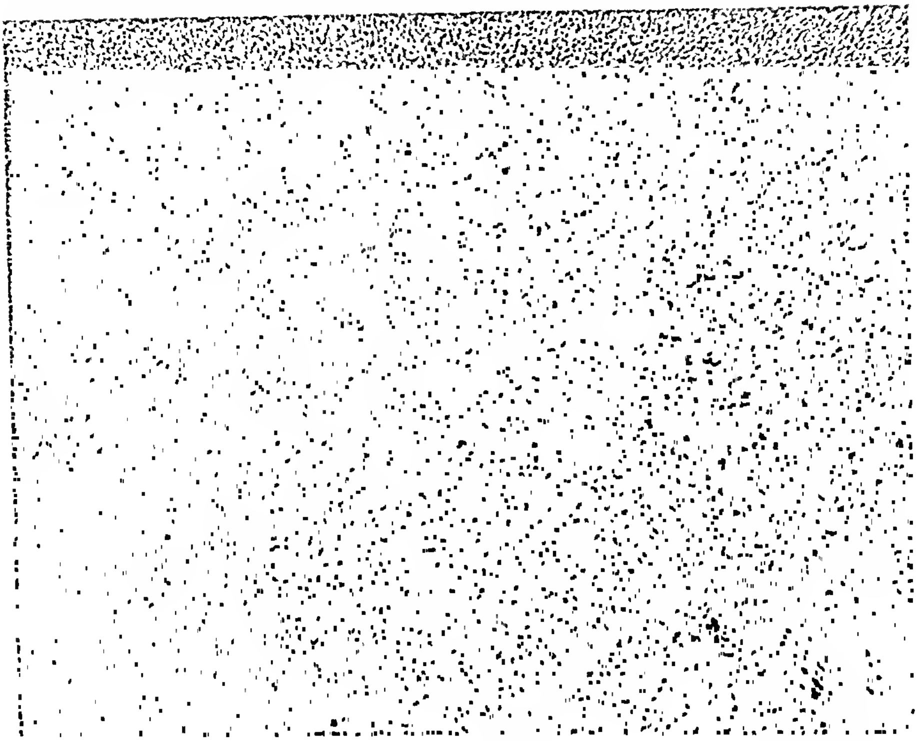


Fig. 5.—Section of a normal blood clot. Note the densely packed erythrocytes (×120).

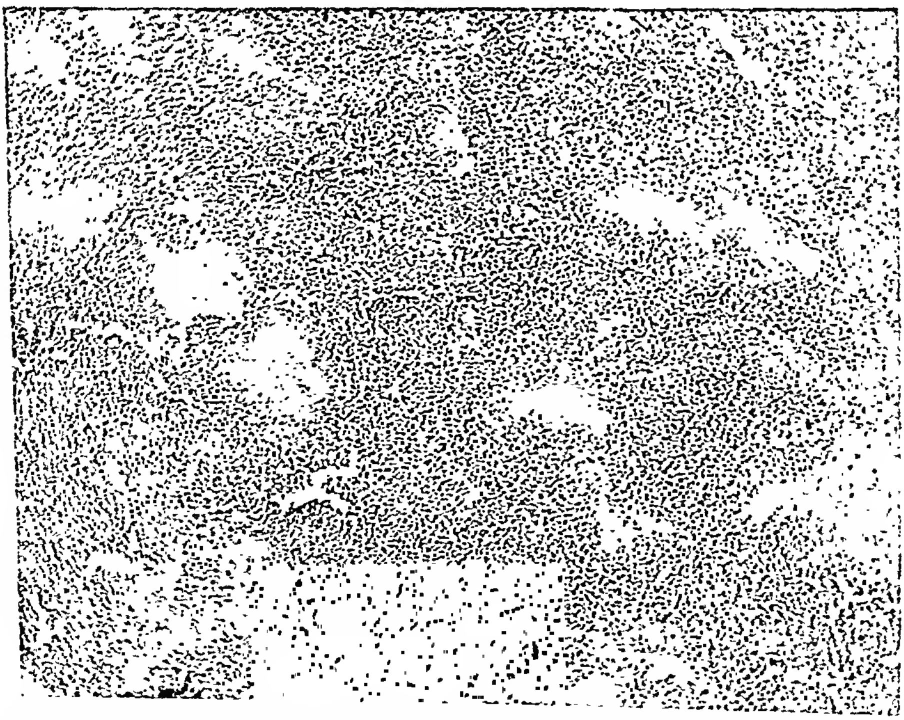


Fig. 6.—Section of a poorly retracting clot from a patient with thrombocytopenia. Note the fragmentation of the clot and the inclusion of large pools of fluid within the clot (×120).

more, unless the effect of the volume of the cells is appreciated, normal retraction may appear to be deficient in patients suffering from erythremia or leucemia.

In this study a highly significant relationship was found between diminished clot retraction and reduction in the platelet count. Tocantins[4] has made direct observations on the action of platelets in the mechanism of retraction of the blood clot. He states: "In the coagulation of blood from man and dog, soon after fibrin is laid down, *intact* platelets in the interior of the mass converge toward the fibrin needles, adhere to them and form large knots at their intersections. It appears that it is by thus strengthening the fibrin framework that platelets help to render the clot more rigid, firm and elastic. As the knots are being formed, the fibrin becomes bent, twisted and shortened. It is perhaps *while* this is going on and partly as a result of it, that the clot undergoes the visible reduction in volume (syneresis)." Furthermore, Tocantins[5] noted complete inhibition of blood clot retraction when antiplatelet serum was added to normal blood in vitro.

Two of us (P. M. A. and S. P. L.)[6, 7] have found that a significant correlation exists between the prothrombin concentration and the *clot retraction index* in patients suffering from obstructive jaundice and a variety of diseases affecting the liver. Boyce and McFetridge[1] noted that the prothrombin concentration was often diminished when the *serum volume index* was low. In the present study we have likewise observed in a significant number of patients suffering from a variety of diseases, an increase in the extracorpuscular volume of the clot when the prothrombin concentration was 50 per cent or lower. The reason for the frequent association of a diminished prothrombin concentration with deficient clot retraction may be due to incomplete conversion of fibrinogen to fibrin. The influence of the platelets on the degree of clot retraction is much greater than that of the prothrombin. It is possible that variations of the number of platelets within the normal range may be a factor in determining the degree of clot retraction, both in normal subjects and in those with hypoprothrombinemia.

Thrombocytopenia and hypoprothrombinemia are not the only factors concerned in clot retraction. We have observed patients in whom the prothrombin concentrations and the platelet counts were normal and in whom clot retraction was deficient; and conversely, we have studied patients in whom the prothrombin or platelet values were low and in whom the blood clots retracted normally. The recent observation of Rabinowitz[8] on the effect of amino acids on clot retraction may be of considerable aid in the solution of these problems.

SUMMARY

The blood clot is composed of cells, fibrin meshwork, and serum. The extracorpuscular volume of the clot is defined as that volume of the clot exclusive of its contained packed cells expressed as a percentage of the total volume of the specimen studied. Presumably, it is composed of serum occluded within the clot and a negligible amount of fibrin. The measurement of the extracorpuscular volume of the clot is a most significant factor in the analysis of the efficiency of blood clot retraction. In one hundred normal subjects the extracorpuscular mean volume measured 9.1 per cent and the standard deviation of this sample was 7.5 per cent; in 42 subjects who had prothrombin concentra-

tions of 50 per cent or less, the mean value was 18.2 per cent and the standard deviation was 12.4 per cent; and in 35 subjects, all of whom had platelet counts of 200,000 or less, the mean value was 38.9 per cent and the standard deviation of the sample was 14.1 per cent. We have arbitrarily considered the normal value to fall within two standard deviations of the mean (–5.9 to 24.1 per cent). Using this criterion, blood clot retraction was diminished in 13 of 42 patients who had hypoprothrombinemia, and in 30 of 35 patients who had thrombocytopenia.

REFERENCES

1. Boyce, F. F., and McFetridge, E. M.: A Serum Volume Test for the Hemorrhagic Diathesis in Jaundice, J. Lab. & Clin. Med. 23: 202, 1937.
2. Boyce, F. F., and McFetridge, E. M.: The Serum Volume Test for the Hemorrhagic Diathesis in Jaundice: Further Observations, New Orleans M. & S. J. 91: 357, 1939.
3. Macfarlane, R. G.: A Simple Method for Measuring Clot Retraction, Lancet 1: 1199, 1939.
4. Tocantins, L. M.: Platelets and the Structure and Physical Properties of Blood Clots, Am. J. Physiol. 114: 709, 1936.
5. Tocantins, L. M.: Technical Methods for the Study of Blood Platelets, Arch. Path. 23: 850, 1937.
6. Aggeler, P. M., and Lucia, S. P.: Relation Between Prothrombin Concentration and Retraction of the Blood Clot, Proc. Soc. Exper. Biol. & Med. 42: 599, 1939.
7. Aggeler, P. M., and Lucia, S. P.: The Bleeding Tendency in Diseases of the Liver and Biliary Passages, Acta med. Scandinav. 107: 179, 1941.
8. Rabinowitz, H. M.: A Study of the Role of Amino Acids in Clot Retraction, Am. J. Surg. 51: 366, 1941.
9. Mettier, S. R.: Personal communication. Unpublished data.

DRY BUFFER SALT MIXTURES FOR USE IN BIOLOGICAL PREPARATIONS*

George A. Hunt, M.S., Ph.D., St. Louis, Mo.

In the preparation of many biological products the procedures specify the adjustment of a product to a given hydrogen-ion concentration. In many such procedures the accuracy of such an adjustment need not be great, provided the pH of the product does not exceed a given limit. Poorly buffered systems sometimes require time and considerable care to avoid exceeding the desired end point.

We have employed preparations of dry buffer salts which are useful, stable, and comparatively accurate. As we have not seen descriptions of dry salt preparations such as we use, and since these mixtures are very convenient, it seems worth while to describe them.

Sodium or potassium phosphate buffers were selected for pH values from 5 to 8. One per cent aqueous solutions of the salts selected for use were prepared and one solution was carefully titrated against the other in the presence of an indicator. The ratios of the buffer salts in solutions giving desired pH values were noted, and the amounts of the original dry buffer salts were determined. The solid buffer salts were powdered in a mortar, then weighed and mixed carefully in ratios determined by the titration, a different mixture

*From the Snodgras Laboratory of Pathology and Bacteriology, City Hospital, St. Louis. Received for publication, April 22, 1942.

being prepared for every pH value desired. For use, approximately 1 per cent of the dry buffer mixture of the required pH was dissolved in the product to be buffered.

In our work we wished to employ two buffer mixtures, one of pH 7.0 and the other of pH 7.6. Five cubic centimeters of 1 per cent $NaH_2PO_4.H_2O$ with 10 c.c. of 1 per cent Na_2HPO_4 (anhydrous) gave a pH value of 7.0, and 5 c.c. of 1 per cent $NaH_2PO_4.H_2O$ with 40 c.c. of 1 per cent Na_2HPO_4 (anhydrous) gave a pH of 7.6. Thus one part of powdered $NaH_2PO_4.H_2O$ mixed with two parts of powdered Na_2HPO_4 (anhydrous) gave a mixture which when dissolved in water produced a pH 7.0. Likewise, one part of the former salt and eight parts of the latter was used for a mixture giving a pH 7.6. These mixtures were prepared in 500 Gm. lots and stored in tightly stoppered bottles.

Two examples may serve to show some of the ways we have employed these mixtures. In the culture of amoeba[1] it was found that the presence of buffers was necessary for successful propagation using the method described by Tsuchiya.[2] A simple method of producing small amounts of broth buffered at pH 7.0, was to add a loopful of the pH 7.0 dry buffer mixture to each culture tube whenever excessive acid was formed in the culture.

In preparing Price-type gonococcus antigen for the complement fixation reaction according to the directions of Torrey,[3] it is necessary to dissolve the trichloroacetic acid precipitated fraction in saline and sodium hydroxide. The final pH of the product should not exceed 9.5 or the antigenic value depreciates.[4] It is easier to prepare an active, stable product by adding a small amount of dry buffer mixture of pH 7.6 to a saline suspension of the precipitate than to add sodium hydroxide carefully drop by drop to the same end point.

The greatest convenience of the dry buffer salt mixture lies in its ability to produce sufficiently constant pH value with widely varying concentrations of the mixture. The constituents of the mixture should be powdered, accurately weighed, and carefully mixed. When the mixture has once been prepared, it is often sufficient to estimate roughly the amount of the mixture needed and to add the dry mixture to the product to be buffered directly from the stock bottle without weighing.

REFERENCES

1. Russi, S., and Hunt, G. A.: A Note on the Cultivation of E. histolytica, J. Bact. 39: 751 (abstr.), 1940.
2. Tsuchiya, H.: Further Studies on the Cultivation of Endameba histolytica. A Complement Fixation Test for Amebiasis, J. Lab. & Clin. Med. 19: 495, 1934.
3. Torrey, J. C., with technical assistance of Montu, E.: A Comparative Study of Antigens for the Gonococcal Complement Fixation Test, J. Immunol. 38: 413, 1940.
4. Price, I. N. O.: The Complement Fixation Test for Gonorrhoea. A New Technique and Its Clinical Application, London County Council Publication, London, 1935, King and Sons, Ltd.

A COMPARISON OF THE RYTZ, KAHN, AND COMPLEMENT FIXATION TESTS FOR SYPHILIS IN A LARGE SERIES OF ADULT MALES

Lewis E. Nolan, M.D., Montgomery, W. Va.

The Rytz test is a rapid, economical precipitation method for the serologic diagnosis of syphilis. The purpose of this paper is to compare the results of 18,681 Rytz, standard Kahn, and complement fixation tests on 5,644 adult male patients, examined over a period of eighteen months, and to determine the accuracy and value of the tests. All case records were carefully reviewed.

The directions given by Rytz[1] were rigidly adhered to in the performance of this test, the Kahn[2] test was performed according to instructions, and the complement fixation test was performed according to the modification of Matz.[3]

For purpose of comparison the Rytz test is read positive or negative; the Kahn test as positive, doubtful, and negative; and the complement fixation test as positive and negative, as recommended by the Committee on the Evaluation of Serodiagnostic Tests for Syphilis of the United States Public Health Service, cooperating with the American Society of Clinical Pathologists.*

Of the 5,644 males tested 98.9 per cent were of the Caucasian race, and 1.1 per cent were colored. The ages ranged from 23 to 95, with an average of 48 years. Each patient received a Rytz, Kahn, and complement fixation test. Some patients received two or more tests, accounting for the total of 18,681 tests.

The patients were divided into four groups: group I: history and clinical evidence of syphilis with positive serology; group II: history and clinical evidence of syphilis with negative serology; group III: no history or clinical evidence of syphilis but with positive serology; group IV: no history or clinical evidence of syphilis with negative serology.

Group I: A total of 846 tests were performed on 149 patients. The Rytz test was positive in the case of 144 persons, or 96.6 per cent; the Kahn test in 137,† or 91.9 per cent; and the complement fixation test in 133, or 89.2 per cent. The Rytz test was positive and the complement fixation and Kahn tests were negative in one case; the Rytz test was negative and the complement fixation test was positive in 3 cases; the Kahn test was positive and the Rytz and complement fixation tests were negative in 2 cases; the Kahn test negative and the complement fixation and Rytz tests were positive in no instances; the complement fixation test was negative and the Kahn and Rytz tests were positive in 7 cases; and the Wassermann test was positive and the Kahn and Rytz tests were

*Complement fixation test: four plus, three plus, two plus, one plus, all recorded positive; plus-minus, doubtful. Kahn test: four plus, three plus, two plus, all recorded positive; one plus and plus-minus, doubtful.

†In addition, doubtful in 8 cases.

Received for publication, April 16, 1942.

being prepared for every pH value desired. For use, approximately 1 per cent of the dry buffer mixture of the required pH was dissolved in the product to be buffered.

In our work we wished to employ two buffer mixtures, one of pH 7.0 and the other of pH 7.6. Five cubic centimeters of 1 per cent $NaH_2PO_4.H_2O$ with 10 c.c. of 1 per cent Na_2HPO_4 (anhydrous) gave a pH value of 7.0, and 5 c.c. of 1 per cent $NaH_2PO_4.H_2O$ with 40 c.c. of 1 per cent Na_2HPO_4 (anhydrous) gave a pH of 7.6. Thus one part of powdered $NaH_2PO_4.H_2O$ mixed with two parts of powdered Na_2HPO_4 (anhydrous) gave a mixture which when dissolved in water produced a pH 7.0. Likewise, one part of the former salt and eight parts of the latter was used for a mixture giving a pH 7.6. These mixtures were prepared in 500 Gm. lots and stored in tightly stoppered bottles.

Two examples may serve to show some of the ways we have employed these mixtures. In the culture of amoeba[1] it was found that the presence of buffers was necessary for successful propagation using the method described by Tsuchiya.[2] A simple method of producing small amounts of broth buffered at pH 7.0, was to add a loopful of the pH 7.0 dry buffer mixture to each culture tube whenever excessive acid was formed in the culture.

In preparing Price-type gonococcus antigen for the complement fixation reaction according to the directions of Torrey,[3] it is necessary to dissolve the trichloroacetic acid precipitated fraction in saline and sodium hydroxide. The final pH of the product should not exceed 9.5 or the antigenic value depreciates.[4] It is easier to prepare an active, stable product by adding a small amount of dry buffer mixture of pH 7.6 to a saline suspension of the precipitate than to add sodium hydroxide carefully drop by drop to the same end point.

The greatest convenience of the dry buffer salt mixture lies in its ability to produce sufficiently constant pH value with widely varying concentrations of the mixture. The constituents of the mixture should be powdered, accurately weighed, and carefully mixed. When the mixture has once been prepared, it is often sufficient to estimate roughly the amount of the mixture needed and to add the dry mixture to the product to be buffered directly from the stock bottle without weighing.

REFERENCES

1. Russi, S., and Hunt, G. A.: A Note on the Cultivation of E. histolytica, J. Bact. 39: 751 (abstr.), 1940.
2. Tsuchiya, H.: Further Studies on the Cultivation of Endameba histolytica. A Complement Fixation Test for Amebiasis, J. Lab. & Clin. Med. 19: 495, 1934.
3. Torrey, J. C., with technical assistance of Montu, E.: A Comparative Study of Antigens for the Gonococcal Complement Fixation Test, J. Immunol. 38: 413, 1940.
4. Price, I. N. O.: The Complement Fixation Test for Gonorrhoea. A New Technique and Its Clinical Application, London County Council Publication, London, 1935, King and Sons, Ltd.

A COMPARISON OF THE RYTZ, KAHN, AND COMPLEMENT FIXATION TESTS FOR SYPHILIS IN A LARGE SERIES OF ADULT MALES

Lewis E. Nolan, M.D., Montgomery, W. Va.

The Rytz test is a rapid, economical precipitation method for the serologic diagnosis of syphilis. The purpose of this paper is to compare the results of 18,681 Rytz, standard Kahn, and complement fixation tests on 5,644 adult male patients, examined over a period of eighteen months, and to determine the accuracy and value of the tests. All case records were carefully reviewed.

The directions given by Rytz[1] were rigidly adhered to in the performance of this test, the Kahn[2] test was performed according to instructions, and the complement fixation test was performed according to the modification of Matz.[3]

For purpose of comparison the Rytz test is read positive or negative; the Kahn test as positive, doubtful, and negative; and the complement fixation test as positive and negative, as recommended by the Committee on the Evaluation of Serodiagnostic Tests for Syphilis of the United States Public Health Service, cooperating with the American Society of Clinical Pathologists.*

Of the 5,644 males tested 98.9 per cent were of the Caucasian race, and 1.1 per cent were colored. The ages ranged from 23 to 95, with an average of 48 years. Each patient received a Rytz, Kahn, and complement fixation test. Some patients received two or more tests, accounting for the total of 18,681 tests.

The patients were divided into four groups: group I: history and clinical evidence of syphilis with positive serology; group II: history and clinical evidence of syphilis with negative serology; group III: no history or clinical evidence of syphilis but with positive serology; group IV: no history or clinical evidence of syphilis with negative serology.

Group I: A total of 846 tests were performed on 149 patients. The Rytz test was positive in the case of 144 persons, or 96.6 per cent; the Kahn test in 137,† or 91.9 per cent; and the complement fixation test in 133, or 89.2 per cent. The Rytz test was positive and the complement fixation and Kahn tests were negative in one case; the Rytz test was negative and the complement fixation test was positive in 3 cases; the Kahn test was positive and the Rytz and complement fixation tests were negative in 2 cases; the Kahn test negative and the complement fixation and Rytz tests were positive in no instances; the complement fixation test was negative and the Kahn and Rytz tests were positive in 7 cases; and the Wassermann test was positive and the Kahn and Rytz tests were

*Complement fixation test: four plus, three plus, two plus, one plus, all recorded positive; plus-minus, doubtful. Kahn test: four plus, three plus, two plus, all recorded positive; one plus and plus-minus, doubtful.

†In addition, doubtful in 8 cases.

Received for publication, April 16, 1942.

negative in 3 cases. In only 6 cases, or 4 per cent of group I, is one test positive and the other two negative. It is evident that the Rytz and Kahn tests more closely agree than the precipitation tests and the complement fixation test.

In group II, 191 patients with history and clinical evidence of syphilis, many of them with previous records of positive serology, had received intensive antisyphilitic therapy and were discharged with seronegative findings, and at the time of this survey were negative serologically.

In group III careful study failed to elicit any history or clinical evidence of syphilis; however, 39 patients had positive serology by one or more of the three tests used. The Rytz test was positive in 27 patients; the Kahn test, positive in 19, doubtful in 7; and the complement fixation test, positive in 32. In this group again the results of the Kahn and Rytz tests were in close agreement, whereas the complement fixation was not in accord with the precipitation tests in 4 cases. The patients in this group had diagnoses of pneumonia, bronchitis, arthritis, appendicitis, undulant fever, duodenal ulcer, tuberculosis, pulmonary, renal calculus, and carcinoma. If the cases in this group are regarded as false-positive reactions, the percentage of the total number of patients tested is 0.4 per cent for the Rytz, 0.4 per cent for the Kahn, and 0.5 per cent for the complement fixation test.

In group IV, 5,265 patients gave no history or clinical findings of syphilis, and the serology was negative.

CONCLUSIONS

1. In a group of 149 syphilitic patients the Rytz test was positive in 96.6 per cent; the Kahn test, positive in 91.9 per cent and doubtful, or one plus, in 5.3 per cent; and the complement fixation test, positive in 89.2 per cent.

2. The so-called false-positive reactions in a group of 5,644 patients were 0.4 per cent for the Rytz, 0.4 per cent for the Kahn, and 0.5 per cent for the complement fixation test.

3. The Rytz test was found to give accurate results when compared with the Kahn and complement fixation tests and should be more widely recognized because of accuracy, speed, and economy in performance of tests for serodiagnosis of syphilis.

REFERENCES

1. Rytz, F.: J. Lab. & Clin. Med. 21: 934, 1936.
2. Kahn, R. L.: The Kahn Test, A Practical Guide, Baltimore, Williams & Wilkins Co.
3. The Wassermann Test—Clin. Bull. No. 10, U. S. Vet. Bur. M., Service by Philip B. Matz, M.D., Feb. 6, 1926.

BLOOD PLASMA PIPETTE*

W. EARL MATTOX AND JOHN A. LEIGHTY, PH.D., INDIANAPOLIS, IND.

IN THE course of our work of preparing large amounts of dried human plasma for the armed forces of the United States, it became necessary to develop a pipette for the separation of plasma from cells, which not only would be rapid, but also would give a high yield of plasma. In most pipettes used heretofore, it has not been possible to draw the plasma rapidly, and at the same time be able to remove it down to within a few millimeters of the junction between the cell layer and plasma layer without also pulling cells up with the plasma.

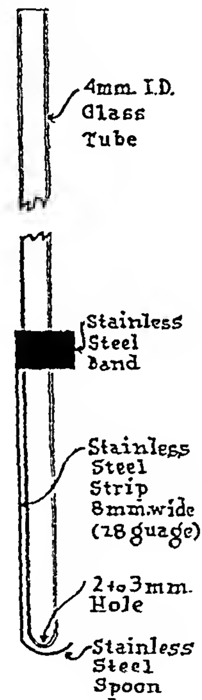


Fig. 1.

Fig. 1 shows a type of pipette which we are now finding very useful in large scale separation of plasma from cells. The stainless steel spoon and holder is made in one piece, and can be moved up and down on the glass tube. This pipette is most efficient when the end of the glass tube is just about touching the spoon.

*From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.
Received for publication, January 21, 1942.

A TECHNIQUE FOR THE EVEN DISTRIBUTION OF GASES THROUGH BACTERIAL CULTURES*

GREGORY SHWARTZMAN, M.D., AND WILLIAM BIERMAN, M.D.
NEW YORK, N. Y.

NUMEROUS workers have noted the influence of various gases upon the growth of microorganisms. These include Wherry and Oliver[1] (1916), Chapin[2] (1918), Kohman[3] (1919), Rendiger[4] (1919), and Rockwell and McKhann[5] (1921), who observed that the growth of meningococci and gonococci was best when the medium was exposed to 10 per cent carbon dioxide. Novy, Roehm, and Soule[6] made a thorough investigation of microbic respiration (1925), and Novy and Soule[7] (1925) studied in detail the respiration of *B. tuberculosis.* Robertson and Sia[8] (1924) determined the growth-inhibitory and bactericidal action of sera and leucocytes upon pneumococcus at different rates of rotation of tubes during incubation. Soule[9] (1928) described the influence of hydrogen and nitrogen upon bacterial growth. Magoon and Brunstetter[10] (1930) devised a special apparatus for the bubbling of gas through media.

We arranged a relatively simple apparatus for the passing of gas through broth cultures held within a water bath. Four large test tubes (25 by 200 mm., 1 inch by 8 inches, 50 ml. capacity) were suspended in a constant temperature water bath.[11] This number was used because it was found most convenient for our studies on the effect of various concentrations of the sulfonamides at different temperatures on the growth of *Streptococcus viridans.* A smaller or larger number could have been employed as well. Each tube, containing 30 c.c. of a 0.4 per cent glucose infusion broth with a pH 7.4, was inoculated with 1 c.c. of an eighteen-hour-old broth culture of *Streptococcus viridans.* The cultures maintained were kept at temperatures ranging from 37.5° C. to 41.5° C. The temperature of the water bath was held constant to within ± 0.05° C. by heaters, thermostat, and stirrer.

After a series of trials a gas mixture, consisting of 10 per cent carbon dioxide, 5 per cent oxygen, and 85 per cent nitrogen, was found favorable for the growth of *Streptococcus viridans.*† A tank containing these gases was connected to a regulator with a pressure gauge and a four-way outlet. From each of these outlets the gases flowed through separate wash bottles to the culture tubes. After bubbling through the cultures, the gas was permitted to pass through another series of wash bottles and then into the air. The constant slow agitation of the culture medium prevented the settling of the bacteria on the

---

*From the Laboratories of Bacteriology and Physical Therapy, the Mount Sinai Hospital, New York.

Received for publication, April 24, 1942.

†A similar mixture was employed by Longsworth and MacInnes[12] in studies on the growth of *Lactobacillus acidophilus* at varying pH and temperature ranges.

bottom of the tube and insured an equal exposure of the organisms to the surrounding environment. The gas within the culture tube coming from the tank as well as that due to the metabolic activity of the organisms, passed continuously through the sidearm into the exhaust gas bottle.

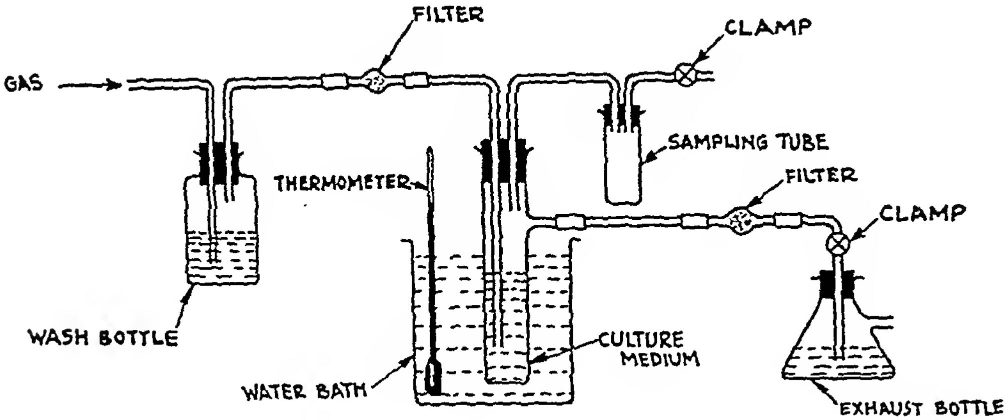


Fig. 1.—Gas coming from the tank passes through a wash bottle, then through a filter into the culture medium. The tube containing the medium is immersed in a water bath, the temperature of which is held constant by heating units, thermostat, and stirrer. The gas passes from the tube containing the culture medium through a side opening into an exhaust wash bottle from which it goes into the air. Closing the clamp on the tube going to the exhaust wash bottle causes the pressure within the culture tube to be raised, forcing the medium through another opening into the sampling tube. After the desired amount of culture medium collects in the sampling tube, the clamp on the tubing coming from it is closed, and the clamp on the tube going to the exhaust bottle is opened to re-establish the previous course of the flow of gas.

When it was necessary to remove a sample of the culture, the opening to an exhaust gas bottle was closed. This caused the building up of gas pressure within the culture tube which forced some of the medium through another opening leading to a sterile sampling tube. A clamp on the opening leading from this tube controlled the inflow. After the desired amount of medium had accumulated in the sampling tube, the outlet from this tube was shut off and the clamp restricting the flow of air into the exhaust bottle was opened.

This technique permitted the removal of the culture sample without contamination of the medium in the large culture tubes. Following the removal of the sample, another empty tube was substituted for it. The sample was thoroughly diluted in 0.04 per cent glucose infusion broth 10^{-6} and 10^{-7}. Poured blood agar plates were then made with the sample variously diluted for bacterial counts.

SUMMARY

A technique is described for the cultivation of microorganisms under the following conditions, namely: (a) constant slow agitation of the cultures during the entire period of growth; (b) carefully controlled temperature; (c) an even distribution of mixture of gases favorable for the microorganisms in the media; and finally, (d) easy removal of samples without any danger of contaminating the culture at any time during the cultivation.

REFERENCES

1. Wherry, W. B., and Oliver, W. W.: Oxygen Tension Requirements of Gonococcus, J. Infect. Dis. 19: 288, 1916.

2. Chapin, C. W.: Carbon Dioxide in the Primary Cultivation of Gonococcus, J. Infect. Dis. 23: 342, 1918.
3. Kohman, E. F.: The So-Called Reduced Oxygen Tension for the Growing Meningococcus, J. Bact. 4: 571, 1919.
4. Reudiger, E. H.: Exclusion of Air in the Cultivation of Gonococcus, J. Infect. Dis. 24: 376, 1919.
5. Rockwell, G. E., and McKhann, C. F.: Growth of Gonococcus in Various Gas Environments, J. Infect. Dis. 28: 249, 1921.
6. Novy, F. G., Roehm, H. R., and Soule, M. H.: Microbic Respiration, J. Infect. Dis. 36: 109, 1925.
7. Novy, F. G., and Soule, M. H.: Respiration of Tubercle Bacillus, J. Infect. Dis. 36: 168, 1925.
8. Robertson, O., and Sia, R.: Method for Demonstrating the Growth-Inhibitory and Bactericidal Action of Normal Serum-Leucocyte Mixtures, J. Exper. Med. 39: 219, 1924.
9. Soule, M. H.: Gas Metabolism of Bacteria in Jordon, E. O. and Falk, I. S.: The Newer Knowledge of Bacteriology and Immunology, Chicago, 1928, University of Chicago Press.
10. Magoon, C. A., and Brunstetter, B. C.: New Vessels for the Efficient Aeration of Bacterial Culture in Liquid Media, J. Bact. 19: 415, 1930.
11. Fisher Scientific Company, Constant Temperature Water Bath. Fisher Unitized Water Bath, Catalogue No. 15-444.
12. Longsworth, L. G., and MacInnes, D. A.: Bacterial Growth at Constant pH, J. Bact. 31: 287, 1936.

CHEMICAL

RELATION OF THE CONCENTRATION OF STARCH SUSPENSIONS TO THEIR USE AS SUBSTRATES FOR THE AMYLASE OF THE DUODENAL CONTENTS OF INFANTS*

WILBURT C. DAVISON, M.D., DURHAM, N. C.

IN STUDYING the effect of the amylase of the duodenal contents of infants on the viscosity of starch suspensions in tenth-molar phosphate mixtures,† the successive measurements of viscosity must be plotted in order to determine the rate of change, since the time required to produce a definite amount of change was inversely proportional to enzymic activity; e.g., if 0.1 c.c. of an enzyme preparation required sixty minutes to reduce the initial viscosity 20 per cent, 0.2 c.c. reduced it a similar amount in thirty minutes.[1] On the other hand, the amounts of change in a definite time were not proportional; e.g., regardless of the strength of the enzyme, the viscosity of starch suspensions to which different amounts of amylase had been added, usually but not always, fell to that of the phosphate mixture in two to twelve hours, and then rose nearly to its original viscosity. This secondary increase in viscosity may be due to the viscosity of the end products of starch digestion, e.g., amylocellulose.

As the strength of an enzyme was inversely proportional to the *time* required to effect a definite percentage of change in the viscosity of the substrate but not to the *amount* of change in a definite period of time, an amylase unit was defined as that amount of enzyme which required sixty minutes to reduce the initial viscosity 20 per cent.[1] Sixty minutes and a 20 per cent change were used to define a unit since faster rates were difficult to determine accurately because the frequent measurements of viscosity consumed one to two minutes each, and the enzymic activity was continuing during the observations; slower rates allowed bacterial growth and other sources of errors to occur. Side reactions, which affected the viscosity, occurred with changes greater than 20 per cent in the substrate.

This linear relationship between the velocity of an enzymic reaction and the amount of enzyme used holds only when the amount of enzyme is much smaller than that of the substrate, so that all of it can combine with the latter.[2, 3] With a relative decrease in the concentration of the substrate, the velocity may be proportional to the square root of the concentration of the enzyme, the so-called Schütz and Borissov law,[4] e.g., increasing the concentration of *sucrose* up to 4 or 5 per cent increases the velocity of invertase reactions,[5] but concentrations

*From the Departments of Pediatrics of the Schools of Medicine of the Johns Hopkins University, Baltimore, Md., and Duke University, Durham, N. C.

Received for publication, April 16, 1942.

†All of these studies were made with the same preparation of soluble starch (Merck; made according to Lintner.

above that level decrease the rate of hydrolysis.[6] With colloidal substrates, as in the action of trypsin on gelatin, an increase in the concentration of the substrate also reduces the rate of hydrolysis, due either to the increased viscosity or to the products of hydrolysis.[3]

Viscosity determinations in enzymic measurements can be plotted against time in four ways: (1) As the "observed" viscosity (η, the outflow time of 5 c.c. of the starch-enzyme mixture); (2) the "net" viscosity (η-η_o, the outflow time of the buffer solution). (3) the "relative" viscosity (η/η_o), and (4) the "specific fluidity" (η_o/η-1). The resulting curves have the same general shape, and since the first method is simpler and as accurate as the other three, it was used in routine amylase titrations in infants.[7] As several viscosimeters were used in these titrations, the initial "observed" viscosities frequently were different,[4] and, therefore, were expressed as 100 per cent, and the subsequent measurements as percentages of the initial "observed" viscosity.

TABLE I

EFFECT OF THE CONCENTRATION OF STARCH SUSPENSIONS ON THE MEASUREMENT OF AMYLASE, BASED ON THE PERCENTAGE CHANGE IN THE "OBSERVED" VISCOSITY (FIG. 1)

CONCENTRATION OF THE STARCH SUSPENSIONS (%)	OUTFLOW TIME OF 5 C.C. OF THE STARCH SUSPENSIONS (η SEC.)	OUTFLOW TIME OF 5 C.C. OF THE BUFFER SOLUTION (η_o SEC.)	RELATIVE VISCOSITY η/η_o	TIME REQUIRED FOR A 20% REDUCTION IN THE INITIAL "OBSERVED" VISCOSITY (SEC.)	AMYLASE UNITS*
7.5	385.2	33.0	11.67	16.5	3.6
7.5	351.4	29.5	11.91	16.5	3.6
5.0	257.7	49.7	5.18	10.5	5.6
5.0	248.0	48.0	5.17	10.5	5.6
2.5	90.5	41.3	2.19	19.0	3.2
2.5	127.4	55.8	2.28	19.0	3.2
1.04	39.2	28.5	1.38	∞	0
1.04	40.6	29.5	1.37	∞	0

*The amount of enzyme which requires sixty minutes to reduce the initial viscosity 20 per cent.[1]

As shown in Table I and Fig. 1, in which the percentage changes in the "observed" viscosity were plotted against time, the addition of the same amount of duodenal contents to starch suspensions of different concentrations produced varied results. In the 1.04 per cent starch suspension the enzymic activity was similar to that in the other concentrations for twenty minutes and then stopped; agglutination occurred at the end of two hours. The rate of the enzymic hydrolysis of 2.5, 5.0, and 7.5 per cent starch suspensions was similar for the first forty minutes, but then the rate of enzymic activity in 2.5 per cent starch practically ceased. The rate of hydrolysis of 5 per cent starch was somewhat faster than that of 7.5 per cent starch for the first one hundred minutes and then became slower. As shown in Fig. 1, the solid and open points of the parallel titrations practically coincided.

After twenty-four hours at 34° C. all the starch-duodenal contents mixtures became agglutinated and their viscosity could not be determined. Some undigested starch remained in the 5 and 7.5 per cent suspensions but not in the

1.04 and 2.5 per cent suspensions after twenty-four hours; i.e., the addition of 5 c.c. of 1:62.5 Gram's iodine to 1 c.c. of the starch-duodenal contents mixtures produced a purplish blue color in the two former, but no color in the last two. All the substrates turned blue when the iodine solution was added before incubation with the duodenal contents.

As the times required for a 20 per cent reduction in the "observed" viscosity of 2.5, 5.0, and 7.5 per cent starch concentrations were more closely related than that for the hydrolysis of 1.04 per cent starch, the higher concentrations were used in other viscometric measurements of amylase.[7] A suspension with a relative viscosity of approximately 2.5 (Table I) was the easiest to work with as clogging of the viscosimeters occurred frequently with the heavier suspensions. Furthermore, the 7.5 per cent suspension became caramelized when autoclaved.

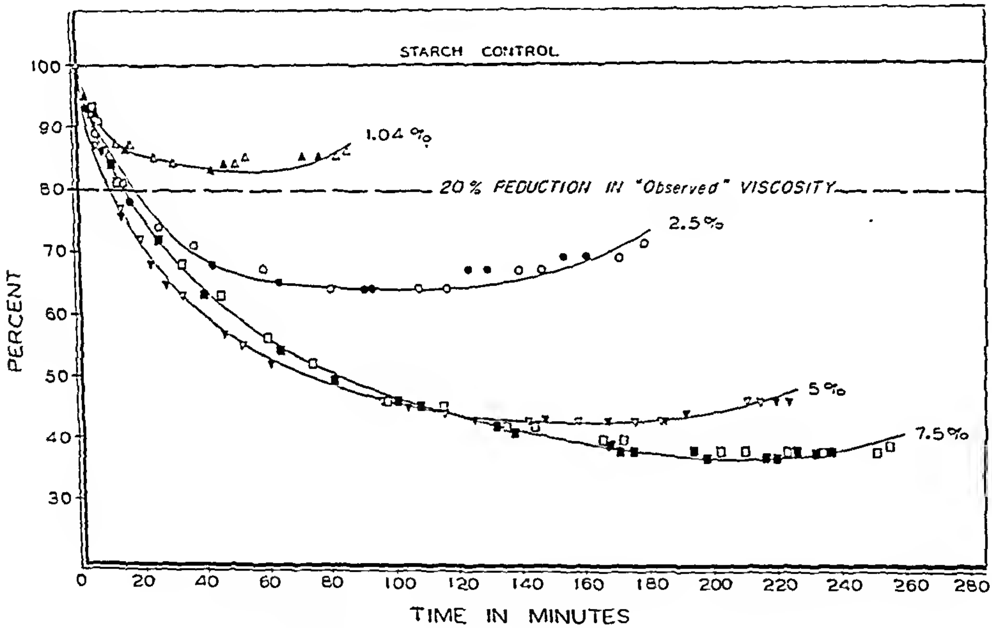


Fig. 1.—Effect of the concentration of starch suspensions on the measurement of amylase, based on the "observed" viscosity. One-tenth cubic centimeter of the same specimen of duodenal contents (diluted 1:20 with normal saline) was added to 10 c.c. of different starch concentrations, and the amylase activity was titrated viscometrically[1] in duplicate. The initial outflow times of the [illegible] tents mixtures in seconds (Table I) were plotted as 100 per cent (initial [illegible]), and subsequent measurements as percentages of this initial "observed" vi[illegible]. [illegible] of each pair of parallel titrations was plotted in solid points, and the other in open.

The relative viscosity of starch suspensions varied with the particular lot of soluble starch employed. A 2.5 per cent suspension of the starch used in Table I produced a substrate of this desired "observed" viscosity, but as much as 6 per cent of other samples of starch was required to produce a suspension of similar viscosity.[8] The digestibility of different lots of starch also has been found to vary, probably due to differences in the proportions of α- and β-amyloses and dextrins.[9] More uniform results have been reported by the use of substrates consisting of mixtures of two preparations of starch.[10]

The viscosity of starch suspensions made with cold phosphate mixtures was fairly constant, but boiling them, or preferably autoclaving them, for fifteen

minutes at 15 pounds pressure made the results uniform. The maximum viscosity was obtained by heating starch solutions to 93° C. Higher temperatures reduced the viscosity. However, starch suspensions, which were both boiled and autoclaved, were used routinely because they were more homogeneous and less likely to contain small lumps which clogged the capillary of the Ostwald viscosimeter, than those which were merely heated to 93° C. or boiled or autoclaved. Amylase titrations with boiled or autoclaved starch suspensions were similar.

The viscosity of autoclaved suspensions (without enzymes) of 1.04, 2.5, and 5 per cent starch was constant for twelve hours, but that of 7.5 per cent starch increased steadily for two hours and then remained constant for twelve hours. After twelve hours the viscosity of all the starch suspensions (without enzymes) increased, probably due to an increase in amylocellulose. However, if these starch suspensions were centrifuged after eight days, the viscosity of the supernatant fluid was the same as that of the phosphate mixture, suggesting that no more of the starch had dissolved. Occasionally a preparation was found which decreased in viscosity. Such suspensions, of course, were useless for amylase determinations.

Amylase determinations, employing starch suspensions less than twelve hours old, similarly made with the same preparation of Lintner starch on different days, were comparable, though far from accurate. Irregular results were obtained with older substrates, even though thymol was added to insure sterility. For example, the rate at which duodenal amylase produced a 10 per cent reduction in the viscosity of fresh and of nine-day-old starch suspensions was the same, but the time required to cause a 20 per cent reduction was much longer with the older preparation than with the fresh suspension.

The starch in these suspensions, made with Lintner soluble starch, apparently consisted of microscopically visible starch granules and ultramicroscopic colloidal particles. The latter probably represented the fraction attacked by amylase, for after the enzyme had reduced the initial viscosity of a starch suspension until it was practically the same as that of the phosphate mixture, the number of starch granules was not reduced. Furthermore, if a starch suspension which had been split by amylase was allowed to stand a few hours, the starch granules sank to the bottom of the tube, leaving the supernatant fluid water clear, in contrast to the supernatant fluid of an unsplit suspension which remained opalescent indefinitely, even though diluted with tenth-molar phosphate mixture until its viscosity was the same as that of the starch suspension which was split by amylase. Because of these two types of starch, it was not possible to determine the actual starch content of starch suspensions at any stage in enzymic reactions by merely comparing their viscosity curves with those of suspensions of unsplit starch of known concentrations.[8]

CONCLUSION

A starch suspension with a relative viscosity of approximately 2.5 and less than twelve hours old was the most suitable for the viscometric titration of the amylase of the duodenal contents of infants, but the results were not always accurate. However, viscometry[1] is preferable to other methods.[13]

Sufficient soluble starch (Merck; made according to Lintner) to make a suspension with a relative viscosity of approximately 2.5 (2 to 6 Gm., depending on the preparation) should be added to 100 c.c. of tenth-molar phosphate mixture (40 c.c. M./10 KH_2PO_4 + 60 c.c. M./10 Na_2HPO_4). The suspension should be shaken vigorously until it becomes homogeneous (usually one minute), boiled for one to two minutes, and autoclaved for fifteen minutes at 15 pounds pressure; one or two thymol crystals should be added, and the suspension filtered, while hot, through one thickness of muslin. After it has cooled to about 34° C, 10 c.c. portions should be pipetted into clean test tubes and kept in the water bath for at least one hour and not more than twelve hours before being used. The final reaction of this substrate is pH 6.8 to 7.0, which is optimal for the amylase of the duodenal contents of infants.[11] If the amylase of taka-diastase is being titrated, phosphate mixtures of pH 3.0 to 4.8 should be used.[12] All the titrations should be done with the same lot of soluble starch. Other concentrations of starch have been used and various methods of preparation tried, but the above appeared to be the most satisfactory.

I wish to thank Dr. Elizabeth Torrey (Mrs. J. G. Andrews) for plotting the enzyme curves and calculating the units; Mrs. H. A. King and Mr. E. H. Clark for the drawings; Dr. Hans Neurath, Dr. Paul Gross, Dr. D. G. Hill, Mr. J. H. Neese, and Mr. W. V. Singletary for verifying the calculations.

REFERENCES

1. Davison, W. C.: A Viscometric Method for the Quantitative Determination of Amylase, Bull. Johns Hopkins Hosp. 37: 281, 1925; Thompson, W. R., Johnson, C. E., and Hussey, R. G.: A Viscosimetric Method of Estimating Enzyme Concentration With Special Reference to Amylase, J. Gen. Physiol. 15: 1, 1931.
2. Waksman, S. A., and Davison, W. C.: Enzymes, Baltimore, 1926, Williams & Wilkins Co.
3. Bayliss, W. M.: The Nature of Enzyme Action, ed. 5, London, 1925, Longmans, Green & Co.
4. Schütz, J.: Eine Methode zur Bestimmung der relativen Pepsinmenge, Ztschr. f. physiol. Chem. 9: 577, 1885; ibid. 30: 1, 1900; Hamburger, F., and Sperk, B.: Untersuchungen über die Magenverdauung bei neugebornen Brustkindern, Jahrb. f. Kinderh. 62: 495, 1905.
5. Nelson, J. M., and Vosburgh, W. C.: Kinetics of Invertase Action, J. Am. Chem. Soc. 39: 790, 1917.
6. Hudson, W. C.: The Inversion of Cane Sugar by Invertase, J. Am. Chem. Soc. 30: 1160, 1908; ibid. 31: 655, 1908; J. Indust. & Engin. Chem. 2: 143, 1910.
7. Davison, W. C.: The Duodenal Contents of Infants in Health, and During and Following Diarrhea, Am. J. Dis. Child. 29: 743, 1925. Idem: Gastric Analyses in Normal Infants and in Those Convalescent From Diarrhea, ibid. 30: 23, 1925. Idem: Difficulties in the Enzymic Titration of Duodenal Contents, Bull. Johns Hopkins Hosp. 70: 504, 1942.
8. Davison, W. C.: Relation of the Concentration of Starch Suspensions to Their Viscosity. In press. Wies, C. H., and McGarvey, S. M.: A Study of the Effects of Certain Variations in Preparation of a Starch Substrate in Amylase Viscosimetry, J. Gen. Physiol. 16: 221, 1932.
9. Chesley, L. C.: The Validity of the Viscometric and Wohlgemuth Methods for the Quantitative Determination of Amylase, J. Biol. Chem. 92: 171, 1931.
10. Thompson, W. R., McGarvey, S. M., and Wies, C. H.: A Reproducible Standard Substrate in Starch-Amylase Viscosimetry, J. Gen. Physiol. 16: 229, 1932.
11. Rothman, P. E., Widener, D. C., and Davison, W. C.: The Optimal Reaction for the Starch-Liquefying Activity of the Duodenal Amylase of Infants, Am. J. Child. Dis. In press.
12. Maslow, H. L., and Davison, W. C.: The Effect of the Hydrogen-Ion Concentration Upon the Starch-Liquefying Activity of the Amylase of Aspergillus oryzae, J. Biol. Chem. 68: 83, 1926. Sherman, H. C., Thomas, A. W., and Baldwin, M. E.: Influence of Hydrogen-Ion Concentration Upon Enzymic Activity of Three Typical Amylases, J. Am. Chem. Soc. 41: 231, 1919.
13. Maslow, H. L., and Davison, W. C.: A Comparison of the Viscometric, Copper Reduction, Polariscopic and Iodometric Methods for Measuring the Rate of Hydrolysis of Starch and Dextrin by Aspergillus oryzae, J. Biol. Chem. 68: 75, 1926.

A METHOD FOR THE DETERMINATION OF CERTAIN SULFONAMIDES IN BILE*

Haddon M. Carryer, M.D., and Arnold E. Osterberg, Ph.D.
Rochester, Minn.

With the increasing use of sulfonamide compounds in the treatment of infection, a knowledge of the excretion of these drugs in the bile has assumed greater importance. A method for determination of sulfanilamide in bile was presented by Ivy and one of us (H. M. C.)[1]; this was a modification of the method of Doubilet. With the recent refinements[2] in chemical procedures employed in the determination of sulfanilamide, and with the introduction clinically of several new drugs in this field, it seems advisable to extend the previously published method in order to include the new improvements and to test the applicability of the method to the new drugs.

We have found the following procedure satisfactory for the quantitative determination of sulfanilamide, sulfapyridine, sulfadiazine, and sulfathiazole in bile. With the last-named drug an additional step must be used.

METHOD

Place 5 c.c. of the bile to be examined in a 50 c.c. volumetric flask. If concentrations of the sulfonamide compound of 2 mg. per 100 c.c. or less are expected, use 10 c.c. of bile. Add 3 c.c. of 2 N potassium hydroxide. Add 3 c.c. of a 40 per cent solution of zinc sulfate drop by drop, with constant agitation. Add portions of hot water and agitate the precipitate to insure solution of the drug. Dilute to approximately 45 c.c. Then add 3 c.c. of normal potassium carbonate. Add a drop of phenolphthalein indicator solution. Dilute when cool to exactly 50 c.c. Filter. Acidify the filtrate exactly to phenolphthalein by the addition of 2 or 3 drops of 4 N hydrochloric acid. By acidifying after filtration, pigmented substances are eliminated by filtration and do not go into solution as they would if the acid were added before they are removed. Dilution of filtrate by acid solution may be considered negligible.

To 10 c.c. of the filtrate add 1 c.c. of tenth-normal hydrochloric acid. Agitate for a moment until no more effervescence is noted. This will prevent interference from bubbles in the photelometer cups in a later stage. Then add 1 c.c. of a freshly prepared 0.1 per cent solution of sodium nitrite. Agitate and allow to stand for three minutes. Add 1 c.c. of a 0.5 per cent solution of ammonium sulfamate. Agitate and allow to stand for two minutes. Add 1 c.c. of a 0.4 per cent aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride. The color developed is read immediately against simultaneously prepared aqueous standards in a colorimeter or, if facilities are available, with a photelometer equipped with a filter.

*From the Mayo Clinic, Rochester.

Received for publication, May 20, 1942.

When sulfathiazole is the drug to be determined, a slight modification of the method is necessary because of the insolubility of the dye product formed. It is necessary to add 5 c.c. of ethyl alcohol to the 10 c.c. portion of filtrate and to the 10 c.c. portion of the standard solution for comparison in the colorimeter before addition of the solution of sodium nitrite. This will insure the solution of dye in subsequent procedures.

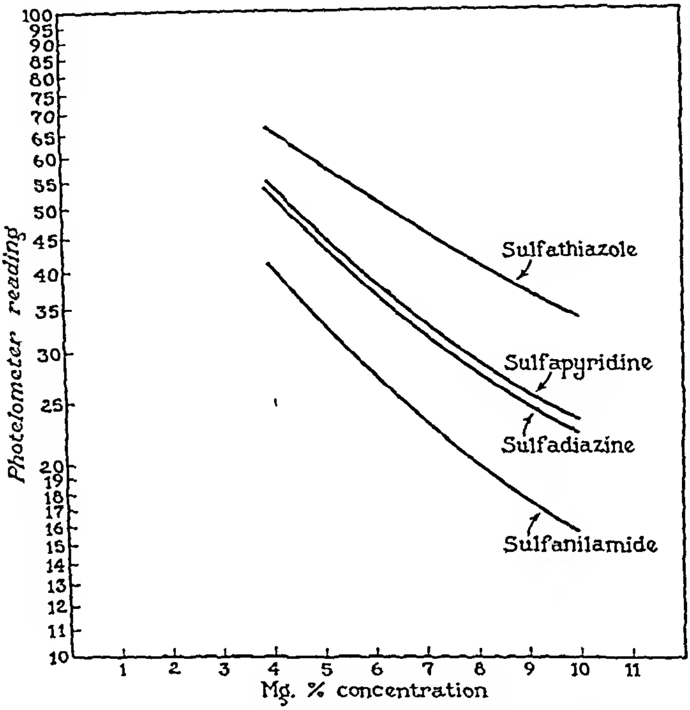


Fig. 1.—Relationship of concentrations of sulfonamides and readings on the photelometer.

This method as described will determine only the drug in its free form; acetylated drug must be hydrolyzed if the total content of drugs is to be found. If this is desired, it is necessary to add to the 10 c.c. of filtrate 0.5 c.c. of 4 N hydrochloric acid and place in a bath of boiling water for one hour. After cooling, make the volume 10 c.c. Proceed as with 10 c.c. of original filtrate with the method described.

Should the colorimeter be used in this determination, the following formula may be employed:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{strength of standard}$$

that is, 0.06 mg. × 100 = concentration of unknown in milligrams per 100 c.c.

We have found the photelometer accurate in this determination when a green filter allowing maximal transmission of 530 Å units is employed. As the dye produced in the reaction has a maximal absorption of 545 Å units,[2] a considerable amount of extraneous color is eliminated suitably. When this filter is employed, the error introduced by the use of aqueous standard rather than bile filtrate will average 1.8 per cent and will not exceed 3 per cent.

Bile which was known to contain 10 mg. per 100 c.c. of the afore-mentioned sulfonamide compounds was used to test the accuracy of the method (Table I). The method is more accurate when sulfanilamide, sulfapyridine, or sulfadiazine is present in the bile than when sulfathiazole is present.

TABLE I

RECOVERY OF SULFONAMIDE COMPOUNDS FROM BILE IN WHICH THE CONCENTRATION WAS KNOWN TO BE 10 MG. PER 100 C.C.

SAMPLE	MILLIGRAMS PER 100 C.C.			
	SULFANILAMIDE	SULFAPYRIDINE	SULFADIAZINE	SULFATHIAZOLE
1	9.7	9.3	9.6	7.7
2	10.0	10.1	10.2	8.1
3	9.7	9.9	9.8	7.8
4	9.5	9.2	9.8	7.3
5	9.7	9.3	9.9	8.0
6				8.5
7				7.8
8				8.1
9				8.0
10				8.6
Average	9.7	9.6	9.8	8.0

The curves from which these data were read are given in Fig. 1. Because of a slight variation in photoelectric cells in these photelometers, a curve should be established for each instrument.

An attempt was made to determine the accuracy of photelometric curves for the sulfonamides on the semilogarithmic graph. By introducing factors to correct for differences in dilution, as in the case of sulfathiazole, and for differences in molecular weight, identical curves should be established. An arbitrarily selected intensity of color was chosen on an abscissa of the graph which was intersected by all curves plotted. Log 4.00 was chosen. The concentration of the drugs at this level of color intensity was then converted by the afore-mentioned factors to terms of concentration of sulfanilamide.

The concentration of all drugs when converted to terms of sulfanilamide and read at the color intensity of log 4.00 varied only slightly. The concentration thus obtained was 3.99 mg. per 100 c.c. ± 0.17 mg. per 100 c.c.

SUMMARY

A method has been presented by which the concentration of sulfanilamide, sulfapyridine, sulfadiazine, or sulfathiazole in bile may be determined. This method has been tested with solutions of known concentration and percentage rates of recovery have been determined.

REFERENCES

1. Carryer, H. M., and Ivy, A. C.: Studies on the Excretion of Sulfanilamide by the Digestive Glands, J. Pharmacol. & Exper. Therap. 66: 302, 1939.
2. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. 128: 537, 1939.

AN OSMOMETER FOR USE WITH SERUM AND ITS SUBSTITUTES*

Henry S. Simms, Ph.D., Raymund L. Zwemer, Ph.D., and
Bertrand E. Lowenstein, M.D., New York, N. Y.

THE present use of "banked blood," stored or dried plasma, and plasma substitutes in the treatment of shock from trauma, burns, or hemorrhage has as its aim the restoration and maintenance of normal blood volume. This aim is accomplished by the injection of solutions which contain, not merely salts and sugars, but also some material which produces a sufficient "protein osmotic" or "oncotic" pressure (erroneously called "colloid osmotic pressure"†) approximately equal to that of normal plasma.

In the case of serum or plasma the "protein osmotic" pressure can be estimated by calculations based on the content of albumin and of globulin. The albumin is responsible for about 80 per cent of the pressure. A rough approximation can be made also from the protein content, as determined by protein nitrogen or plasma specific gravity. However, with dried plasma, gelatin, pectin, or other blood substitutes, the osmotic pressure can best be determined by direct measurement.

The use of a long tube attached to a membrane[1] requires twenty-four hours or longer to reach equilibrium, so that bacterial action and dilution of the serum can cause serious errors. Turner[2] in 1932 devised a method for counterbalancing the pressure so as to reach equilibrium in five to twenty hours. In 1936 Sumalt and Landis[3] constructed an ingenious, but complicated, apparatus which automatically counterbalanced and recorded the pressure until equilibrium was reached. Hepp[4] in 1936 invented a micro-osmometer in which ultrafiltrate from a sample of serum was sucked through a large membrane into a capillary. The balancing pressure was then adjusted until the capillary meniscus was seen through a microscope to be stationary. Peters and Saslow[5] claimed to obtain good results with this osmometer of Hepp. There is, however, the danger that

*From the Departments of Pathology and of Anatomy, College of Physicians and Surgeons, Columbia University, New York.

This work has been aided by grants from W. R. Warner and Company, the Upjohn Company, and the John and Mary R. Markle Foundation.

†We dislike all three of these terms which are applied to the osmotic pressure observed when a nondiffusible ion on one side of a membrane is in equilibrium with diffusible inorganic electrolytes. It was stated by Peters and Van Slyke (Quantitative Clinical Chemistry, Vol. I, page 659) that of the 25 mm. (of mercury) osmotic pressure exerted by blood plasma against isotonic saline solutions, 20 mm. pressure is due to the protein molecules while 5 mm. pressure results from the unequal distribution of diffusible ions according to Donnan's law (Zschr. f. Electrochemie 17: 572, 1911). The latter phenomenon was further studied by Jacques Loeb (J. Gen. Physiol. 3: 667, 691, 827, 1920; 4: 73, 1921).

The term "protein osmotic pressure" is applicable only to systems, such as plasma, where the nondiffusible ions are proteins. The term "colloid osmotic pressure" is particularly objectionable, since it implies that the pressure involves a colloidal phenomenon. Actually the large ion need not be a protein (Donnan used a dye). Perhaps "oncotic pressure" is the least objectionable term in current usage which is applied to the phenomenon. However, this connotes "swelling" and does not distinguish, except by usage, between the observed osmotic pressure when only the large ions are nondiffusible and the theoretical osmotic pressure due to all ions and molecules. We prefer the term "Donnan pressure" but realize that this also is not clearly defined.

the large capillary correction (about 90 mm. of water) and also the use of rubber connection may cause appreciable errors. The errors of the Krogh apparatus were discussed by Wells.[6]

The apparatus herewith described (Fig. 1) was designed to give rapid and accurate results which involve no capillary correction. It uses only 1 to 2 ml. of serum or other fluid, and gives accurate readings within fifteen minutes.

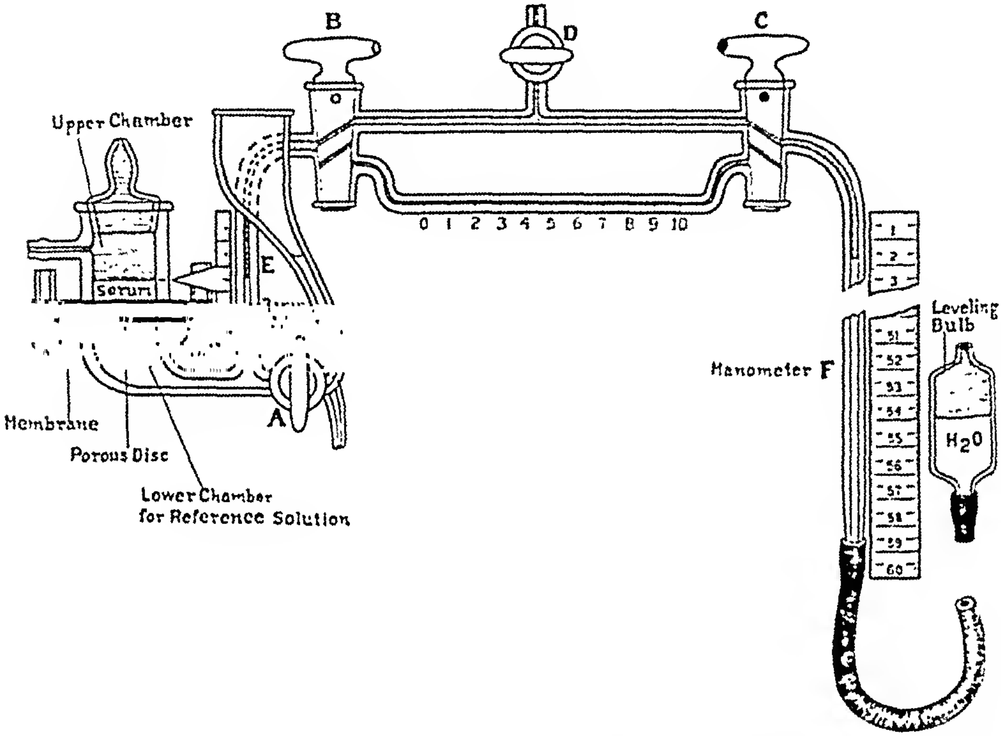


Fig. 1.—The S.Z.L. osmometer. This is firmly supported on a wooden stand not shown in the diagram. Air spaces are shaded, solutions are shown white and the kerosene drop is indicated in black. Detailed description is in the text. The centimeter divisions on the two scales may serve to indicate the dimensions of the apparatus.

DESCRIPTION OF OSMOMETER

In Fig. 1 the osmometer may be seen to consist of the following parts: a removable upper chamber, for the serum or other solution, with an inside diameter of 25 mm. The side arm and the hole in the cover permit the flow of carbon dioxide—air mixture over the solution to maintain pH. The upper chamber is clamped to a lower chamber with the membrane in between glass surfaces which are ground flat. The membrane is supported by a porous disk of fritted pyrex or alundum. The lower chamber is used for the "reference fluid" (such as serum ultrafiltrate or similar physiologic solution), which is introduced from a filling cup through stopcock *A*. The same stopcock serves to drain the chamber. The lower chamber is connected through tube *E* and stopcocks *B* and *C* to the manometer tube *F*, to which a small leveling bulb is attached with heavy walled ⅛ inch rubber tubing. Tubes *E* and *F* should have the same inside diameter (about 3 mm.). Stopcocks *B* and *C* are connected by an upper, 2 mm. bore, tube (having an outside stopcock *D*) and also by a lower capillary tube

in which a drop of colored kerosene serves to indicate motion. The membrane area is 1,000 to 1,700 times the capillary cross section and an observable movement of 0.2 mm. in the capillary represents a displacement of only 0.003 to 0.005 per cent of the serum volume.

METHOD FOR USING OSMOMETER

The osmometer is prepared for use by first cleaning it and then attaching it to a firm board. The leveling bulb is filled with distilled water and is supported by an adjustable clamp. A small drop of kerosene, colored with scarlet red, is introduced into the horizontal capillary tube by removing the plug of stopcock *B* and using a syringe with a bent needle or a dropper with a fine bent tip. By lowering the leveling bulb slowly, the drop is drawn into the tube; it should be 5 to 10 mm. long. No kerosene should remain at the end of the tube near the stopcock.

To fill the osmometer for the zero reading, the porous disk is put in place and the lower chamber is filled with the desired "reference fluid," such as serum ultrafiltrate[7] or a physiologic solution such as is used in these laboratories.[7] This "reference fluid" is introduced from the filling cup through stopcock *A* while stopcocks *B* and *D* are open. A prepared collodion membrane (see below) is then placed over the disk in such a manner as to exclude bubbles of air. The upper chamber is clamped in position and into it 2 or 3 ml. of the "reference fluid" is placed. With *B* and *C* open across the upper tube (and *D* closed) the leveling bulb is lowered to give a negative pressure of about 200 mm. of water for two or three minutes. This pulls the membrane down tight onto the disk. Then stopcock *B* is closed and *D* is opened. The leveling bulb is then raised to bring the water near the top of the manometer tube *F* and stopcock *D* is closed. The "reference fluid" in the vertical tube *E* should be at approximately the level of the fluid in the upper chamber. It can be adjusted by stopcock *A*.

A "zero reading" is taken by opening stopcock *B* and *C* to the lower (capillary) tube and then adjusting the leveling bulb until the drop of kerosene remains stationary for five minutes. If the drop moves to the right, the bulb should be raised. If it moves to the left, the bulb should be lowered. A half minute should elapse after each adjustment before the first observation is made, and the next reading is taken at least a minute later. With a little practice the amount of adjustment required can be estimated from the rate of movement of the kerosene drop. When there has been no movement for five minutes, a record is made of the height of the water column in the manometer above the water surface in the leveling bulb. The height of the "reference fluid" in tube *E above* the fluid surface in the upper chamber is also recorded and *subtracted* from manometer *F* reading to obtain correct "zero reading." If the fluid in *E* is below that in the upper chamber, the difference is added to the manometer reading.

The zero reading should equal zero. Any deviations may be due to an air bubble under the membrane, or a failure to draw the membrane down flat against the disk, or to kerosene at an end of the capillary tube. The zero reading should be repeated after momentarily reversing stopcocks *B* and *C*

and, if desired, readjusting the water level while D is open. With the pressure set at the previous reading there should be no movement of the kerosene drop.

Readings on the unknown solutions are taken next. Stopcock B is closed, the fluid in the upper chamber is drawn off, and the membrane is dried quickly with filter paper. One to 2 ml. of the serum or other unknown solution is introduced immediately. B and C are opened to the upper tube, and the leveling bulb is lowered to approximately the expected pressure. Adjustments are then made, as described above, preferably alternating the adjustments above and below the final reading, thus making sure that there is a real Donnan pressure. When the kerosene drop remains stationary for at least five minutes, the manometer is read. It can be checked by repetition of the procedure.

The membrane can be used for a number of unknown solutions, each solution being removed and the membrane wiped with filter paper before the next solution is placed in the upper chamber. It is well to start with the more dilute solutions and finish with the more concentrated solutions, or else wash the membrane with the "reference fluid" between samples.

Cleaning of the osmometer after use can be done with cleaning solution, which is allowed to stand in the filling cup and lower chamber and is drawn up into tube E. There is less danger of affecting the pH if 0.01 molar sodium hydroxide is used, instead of dichromate cleaning solution, and followed by 0.02 molar acetic acid, rinsing with distilled water after each solution.

Membranes. We obtained best results with membranes made from Merck's C.P. 5 per cent collodion solution on machines previously described.[7] These tubular membranes are cut into 50 to 55 mm. squares or circles and are preserved under distilled water, never being allowed to dry out. For twenty-four hours or more before use, the membranes are allowed to soak in physiologic solution (preferably "Z solution"[7]). This insures saturation of the membranes with calcium and permits a rapid equilibrium which is not otherwise possible. The Viscose Corporation sausage skin and No. 600 DuPont cellophane also gave good results, but these have a pore size of about 2 mμ, which may account for the longer time necessary for a detectable movement of the kerosene bead to appear.

Flat membranes, similar to the first type but less satisfactory, were made by pouring the collodion solution onto mercury in a Petri or similar dish. One milliliter of collodion solution covers 6.5 to 7 sq. cm. of surface. Hence a dish with a diameter of d cm. will require 0.12 d^2 ml. Allow to dry for thirty minutes in a dust-free atmosphere, soak in 50 per cent alcohol for fifteen minutes or longer, then wash with distilled water.

Pore size of the membranes. The osmometer can be used for the measurement of alleged pore size of membranes, as it is commonly rated. Distilled water is placed in both upper and lower chambers, using a membrane which has soaked in distilled water. After a zero reading has been taken, a negative pressure of about 200 mm. (H) is established and the time t in seconds required to move the kerosene drop a given distance L is observed. Using another sample of the same membrane (s mm. square), the thickness l is measured with a micrometer and the water content W is determined by weighing before and after drying. All measurements are in millimeters and the weight is in milligrams.

The pore diameter (in millimicrons) is given by the equation

$$d = \left(56{,}600 \, \frac{d_c}{d_m}\right) l \; s \sqrt{\frac{L}{W \, H \, t}}$$

The ratio of the capillary diameter d_c to the effective membrane diameter d_m can be permanently determined for each apparatus by estimating the capillary diameter as follows: W' milligrams of mercury is introduced into the capillary tube and the length l_c of the column is noted. The diameter in millimeters is then equal to $d_c = 0.307 \sqrt{W'/l_c}$.

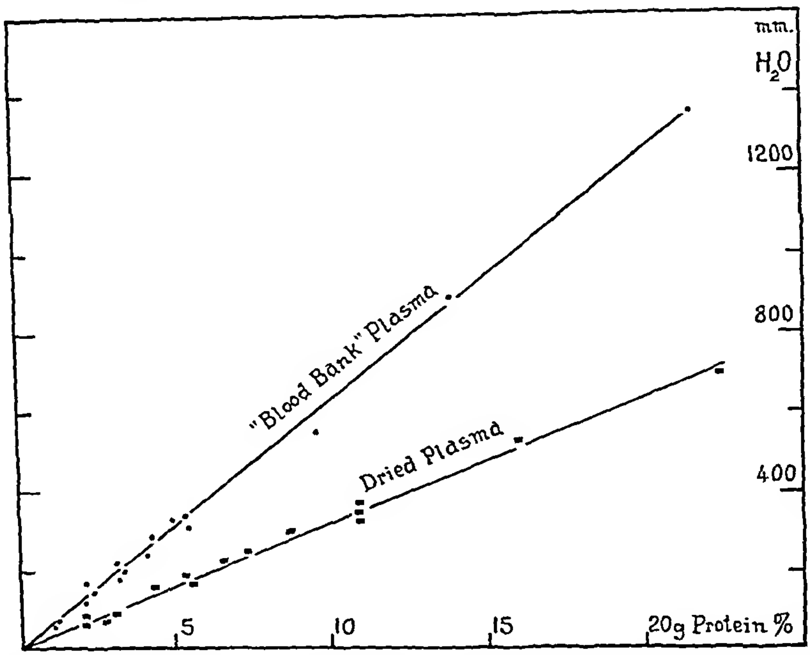


Fig. 2.—The Donnan osmotic pressures of various concentrations of human plasma, plotted against grams of protein per 100 c.c. Upper curve: Citrated human plasma from a "blood bank." The higher concentrations were obtained by dialysis in a collodion bag. Lower curve: Dried human plasma which had been found to be unsatisfactory by other tests. The various concentrations were obtained by weighing the dried powder.

In all cases the protein concentration was checked by the Barbour and Hamilton falling-drop determination of density.

Linear curves were also obtained with dilutions of horse serum, cat plasma, and a sample of approved dried human plasma.

RESULTS

Fig. 2 illustrates the results obtained with this apparatus. The curve for blood plasma at various concentrations above and below the normal level is seen to give consistent values. Another curve in Fig. 2 shows some observations on dried plasma at various concentrations. These values, although lower than for normal plasma, are consistent among themselves. Readings were found to be reproducible within 5 mm. of water.

In addition to the consistency of the readings it will be noted that throughout the range from zero up to four times normal concentration, the osmotic pressure is a linear function of the protein concentration, as measured by specific gravity. This agrees with the statement of Homer Smith and co-workers[8] and is contrary to the claim of Adair, Adair, and Greaves.[9] Further presentation of results will be included in future papers.

SUMMARY

An apparatus for measuring the "protein osmotic" or "oncotic," pressure of serum or other solutions is described. This apparatus requires only one or 2 ml. of fluid; it involves no correction for capillarity and it gives, in fifteen minutes, values which are reproducible within 5 mm. of water.

Rapid equilibrium is produced by exerting a negative pressure on the "outer" or "reference fluid" by means of a leveling bulb. When the equilibrium pressure is found, a drop of kerosene in a horizontal capillary tube becomes stationary. The Donnan osmotic pressure is then read from a manometer.

REFERENCES

1. Kylin, E.: Studien über den kolloidosmotischen Druck, Ztschr. f. d. ges. exper. Med. 70: 683, 1930.
2. Turner, Abbey H.: The Validity of Determinations of Colloid Osmotic Pressure of Serum, J. Biol. Chem. 96: 487, 1932.
3. Sunalt, M., and Landis, E. M.: An Automatic Recording Apparatus for Measurement of Colloid Pressure, J. Lab. & Clin. Med. 22: 402, 1936.
4. Hepp, Oskar: Ein neues Onkometer zur bestimmung des kolloidischen Druckes, etc., Ztschr. f. d. ges. exper. Med. 99: 709, 1936.
5. Peters, E., and Saslow, G., Performance of the Hepp Micro-osmometer, J. Gen. Physiol. 23: 177, 1939.
6. Wells, Herbert S.:, Some Criteria of Accuracy for the Measurement of the Osmotic Pressure of Colloids in Biological Fluids, Am. J. Physiol. 101: 409, 1932.
7. Simms, H. S., and Sanders, M.: Use of Serum Ultrafiltrate in Tissue Cultures for Studying Fat Deposition and for Propagation of Viruses, Arch. Path. 33: 619, 1942.
8. Smith, Homer W. et al.: Glomerula Dynamics in the Normal Human Kidney, J. Clin. Investigation 19: 751, 1940.
9. Adair, G. S., Adair, M. E., and Greaves, R. F. N.: Osmotic Pressure, After Resolution of Serum Dried From the Frozen State, J. Hyg. 40: 548, 1940.

THE MICRODETERMINATION OF GOLD IN BIOLOGICAL TISSUES*

WALTER D. BLOCK, PH.D., AND OLIVER H. BUCHANAN, M.S.
ANN ARBOR, MICH.

IN ORDER to determine small quantities of gold in tissues it was found necessary to modify the procedure described by Block and Buchanan[1] for the microdetermination of gold in blood and urine. The modifications were primarily in the digestion of the tissues, but the final procedure so differed from the original method in details that it is thought advisable to report the complete procedure with typical recoveries for tissues.

PROCEDURE

Digestion.—The tissue (0.5 to 10 Gm.) to be analyzed is placed in an accurately calibrated 100 c.c. Kjeldahl flask whose neck has been shortened to 5 or 6 cm.[1] Ten to 20 c.c. of concentrated nitric acid and a few drops of caprylic alcohol are added and the tissue is allowed to stand for a few hours until solu-

*From the Rackham Arthritis Research Unit, University of Michigan Medical School, Ann Arbor.

The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.

Received for publication, May 25, 1942.

tion has occurred. Ten to 15 c.c. of concentrated sulfuric acid are introduced into the flask which then is heated on a Kjeldahl digestion rack until the tissue is thoroughly charred. After the digest is cooled, a 30 per cent solution of hydrogen peroxide (Superoxol) is added drop by drop until the solution is water clear. The solution is evaporated to dryness over a microburner. The sulfuric acid fumes are removed by inserting into the neck of the flask a glass tube which is connected through a water trap to a water pump.[1] The contents of the flask are then treated with aqua regia to convert the gold to the chloride. If the tissues contain amounts of gold less than 200 micrograms, the color is developed and read as previously described[1] directly with the entire contents of the digestion flask. However, if larger amounts of gold are present, the aqua regia solution in the flask is heated to boiling, cooled, and transferred to a 100 c.c. volumetric flask. The Kjeldahl flask is rinsed twice with 15 to 20 c.c. of boiling aqua regia, the washings are added to the volumetric flask, which is made to volume with distilled water. Aliquots containing 50 to 150 micrograms of gold are transferred to the shortened graduated 100 c.c. Kjeldahl flasks and evaporated to dryness. Remaining traces of sulfuric acid are removed as before and the gold is reconverted to auric chloride by boiling with aqua regia. The aqua regia solution of this aliquot is then used to develop the color. Any fatty or muscular tissue (legs, gastrointestinal tract) is treated in the same fashion, except that the digestion is carried out in a 300 c.c. Kjeldahl flask with the addition of selenium as a catalyst. After evaporation to dryness with complete removal of the sulfuric acid, the residue is put into solution by boiling with three 40 c.c. portions of aqua regia. All the washings are collected in a 250 c.c. volumetric flask, made to volume with water, and aliquots containing 50 to 150 micrograms of gold are transferred to the special 100 c.c. Kjeldahl flasks. The procedure is then carried out as already described for other tissues.

This same procedure can be applied to large organs or carcasses of small experimental animals as well as to dried animal and human feces, the only modification being that a 500 c.c. Kjeldahl digestion flask is used.

Development of Color.—This is accomplished[1] by the addition of 75 c.c. of distilled water to the precipitate in the 100 c.c. Kjeldahl flask in order to dissolve the inorganic salts. Three-fourths cubic centimeter of hydrochloric acid (1:4), 8 c.c. of potassium fluoride (10 per cent), and 1 c.c. of o-dianisidine solution (0.1 per cent) are then added to the flask. The volume is made up to 100 c.c. with distilled water, the flask is stoppered and the solution thoroughly mixed. The solution is then transferred to a colorimeter absorption tube, filling the tube to within 1 to 2 cm. of the top. After stoppering, the pink color, which reaches its maximum intensity within three to ten minutes, is read in the Evelyn photoelectric colorimeter with filter 440 and a center setting of 76.

Special attention should be called to the amount of 1:4 hydrochloric acid added in the color reaction, for the rate of color development and fading depends upon the ratio of fluoride to acid. The amount of acid which remains in the flasks after conversion with aqua regia depends upon the amount of inorganic salts present. A maximum color will develop in six to eight minutes if 0.75 c.c. of 1:4 hydrochloric acid is used in direct determination of gold in tissues or urine. In determinations of gold in carcasses or feces 1.25 c.c. of the

acid must be added, and in the case of diluted tissues, 2.5 c.c. It is best to read the color at one-minute intervals during the development until the maximum color is reached and fading begins. Since it requires only fifteen seconds to make a reading, four tubes may be easily read at minute intervals.

TABLE I

RECOVERY OF KNOWN AMOUNTS OF GOLD ADDED TO TISSUES AND EXCRETA OF THE WHITE RAT

	GOLD ADDED	GOLD FOUND			GOLD ADDED	GOLD FOUND	
	mg.	mg.	per cent		mg.	mg.	per cent
Heart	0.010	0.00895	89.5	Whole legs	0.500	0.4683	93.7
	0.020	0.01946	97.3		5.000	4.797	95.9
	0.030	0.02968	98.9		20.000	18.864	94.3
Lung	0.010	0.00928	92.8	Entire carcass	2.000	1.829	91.5
	0.020	0.01973	98.7		10.000	9.870	98.7
	0.150	0.1471	98.1		40.000	39.420	98.5
Spleen	0.020	0.0199	99.5	Urine*	0.300	0.294	98.0
	0.100	0.100	100		5.000	4.813	96.2
	0.200	0.1916	95.8		10.000	10.000	100
Liver	0.200	0.199	99.0	Feces*	0.500	0.4064	81.3
	1.000	0.971	97.1		2.000	1.987	99.3
	5.000	4.855	97.1		5.000	4.920	98.4
Kidney	0.100	0.958	95.8				
	2.000	1.974	98.7				
	5.000	4.855	97.1				

*Gold added to urine and feces collected over a two-week period.

RESULTS

Table I gives the percentage recovery of various amounts of gold added to tissues and excreta of the white rat. The recoveries vary from 90 to 100 per cent, except in one case, in which 0.5 mg. of gold was added to feces. In this instance recovery was 81.3 per cent. This comparatively low recovery is probably associated with the inhibition of color development due to the presence of large amounts of inorganic salts in relation to the small quantity of added gold.

CONCLUSION

The method of Block and Buchanan for the microdetermination of gold in urine and blood has been modified to apply to tissues and feces. Recoveries of added gold by this procedure vary from 90 to 100 per cent.

REFERENCE

1. Block, W. D., and Buchanan, O. H.: The Microdetermination of Gold in Biological Fluids, J. Biol. Chem. 136: 379, 1940.

A STORAGE VESSEL FOR BLOOD*

Frederic E. Holmes, Cincinnati, Ohio

THE slow rate of diffusion of gases into oil delays, but does not entirely prevent, measurable changes in the gas content of specimens stored under oil. The vessel shown in Fig. 1 was used for the anaerobic collection and storage of 10 to 15 ml. specimens of blood "under mercury." It is compact, portable, and stands firmly on its own base. It has proved satisfactory in about eight months' use in this laboratory.

In preparation of the vessel for use, the lower expanded part of the outer bulb, the storage chamber (inner bulb), and the capillary and cup above it are filled with mercury. A piece of ⅛ by 3/32 inch rubber tubing (not shown) is attached to the nipple in the cup and extends about 2 cm. above its rim. Liquid anticoagulant in a graduated 1 ml. pipette inserted in the top of this tube is allowed to flow toward the outer bulb until it passes through the stopcock. Then the stopcock is turned and the desired amount (usually 0.3 ml.) is delivered into the chamber. The rubber tube is removed and the cock is reopened to allow mercury to fill the capillary between cup and chamber. The rubber tube is again attached and filled with mercury from a capillary pipette or syringe.

The blood specimen is taken into a syringe containing oil. The glass tip of the syringe is inserted into the rubber tube. Any minute bubble trapped at this point is rejected through the capillary leading into the outer bulb. The unexposed specimen is allowed to flow into the chamber. When the rubber tube is removed, mercury in the cup immediately seals the top of the capillary. The cock is opened momentarily to allow mercury to flush blood from the capillary into the chamber. Mercury is swirled around the walls of the chamber by gently shaking the vessel in a slanted position in order to mix the blood with anticoagulant, and later to maintain a uniform distribution of red blood cells in the specimen during sampling. To withdraw a sample for analysis, a clean piece of rubber tubing is attached as before. Mercury from the capillary and a drop or two of blood are sucked into any convenient pipette and discarded. The sample is then drawn into a measuring pipette, such as the Van Slyke-Ostwald. The remaining unused specimen is protected by resealing with mercury in the usual manner.

---

*From the Department of Internal Medicine, College of Medicine, University of Cincinnati.

Received for publication, June 1, 1942.

The dimensions of parts of the apparatus, which are not critical, can be obtained by comparison with the scale included in Fig. 1. For best results the capillary and bore of the plug of the cock must be free of offsets, bulges, or

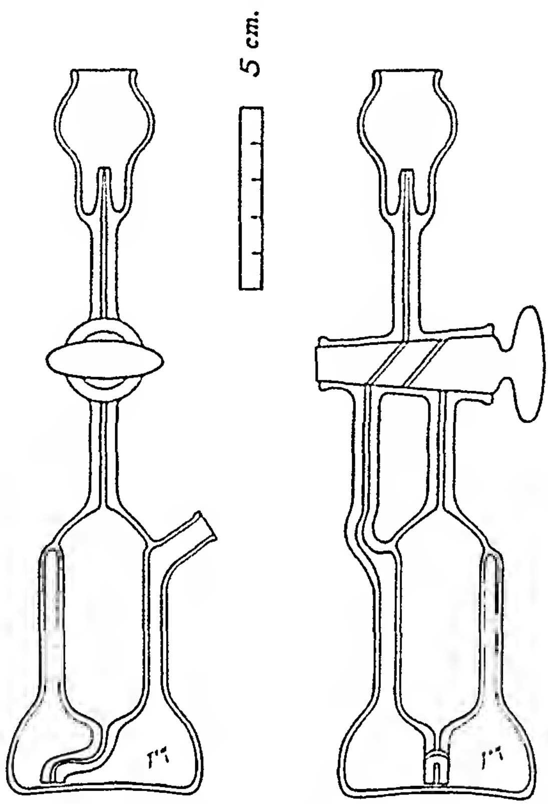


Fig. 1.—Two views of the mercury-sealed blood storage vessel. Left, from the right side. Right, front view.

pockets in which blood can collect, and should be of approximately 0.8 mm. inside diameter. The inside junction of the capillary with the top of the chamber should be abrupt so that the capillary remains filled with mercury during stirring of the specimen.

In Memoriam

ARTHUR H. THOMAS

Few names have been more closely identified with medical laboratories than has that of Arthur H. Thomas whose death occurred on August 31, 1942.

The Thomas catalogue stands on the shelves of nearly all clinical laboratories. It is doubtful whether there is a laboratory in the country which does not have many pieces of diagnostic apparatus manufactured by the Thomas Company. The name Arthur H. Thomas has appeared in the advertising pages of every issue of *The Journal of Laboratory and Clinical Medicine* from Volume 1, Number 1.

A native of Pennsylvania, Mr. Thomas entered the employ of the old Philadelphia firm of James W. Queen and Company, dealers and makers of optical and scientific instruments in 1892. He organized his own company in 1900. Since then, Arthur H. Thomas Company, dealers in laboratory apparatus and reagents, have remained among the leaders in their field.

Mr. Thomas was a Trustee and Director of Bryn Mawr College and a member of the Board of Managers of Haverford College. He was a member of the Board of the Provident Mutual Life Insurance Company.

DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TRYPSIN, Method of Assaying Suitable, for Routine Use in Diagnosis of Congenital Pancreatic Deficiency, Andersen, D. H., and Early, M. V. Am. J. Dis. Child. 63: 891, 1942.

The modified Fermi method is carried out as follows:

The substrate is a 7.5 per cent solution of Knox gelatin, obtained from the local grocer. In a 50 c.c. beaker are placed 18.75 Gm. of gelatin. About 40 c.c. of cold distilled water are gradually added, and the mixture is stirred until it is a semiliquid paste free from lumps. This paste is transferred through a small funnel to a 250 c.c. volumetric flask, and the beaker and funnel are washed with about 100 c.c. of water. The flask is heated on a water bath, with occasional shaking until the solution is clear. It is then cooled, made up to volume with water, and replaced on the water bath until the gelatin is entirely melted. The flask is shaken until the contents appear homogeneous. The gelatin is transferred to a stock bottle, and a small amount of thymol is added. When stored in the refrigerator, the substrate can be used for at least six weeks.

The substrate should be left to melt in an incubator at a temperature of 37° C. for approximately one hour before using. Twenty Wassermann tubes are set in a rack in pairs and labeled from 1 to 10; 2 c.c. of melted substrate are placed in each with a transfer pipette. One cubic centimeter of duodenal juice is placed in a 10 c.c. volumetric flask and made up to volume with a 5 per cent solution of sodium bicarbonate, this giving a 1:10 dilution. One cubic centimeter of this material is placed in a second 10 c.c. flask and made up to volume in the same way, with a resulting 1:100 dilution. Each of the first pair of tubes then receives 0.125 c.c. of the 1:100 dilution, measured with a 1 c.c. Mohr graduated pipette, and 1.875 c.c. of 5 per cent sodium bicarbonate solution: this makes a total volume of 4 c.c. in each tube, of which 0.00125 c.c. is duodenal juice. The remaining tubes are set up in a similar manner; the second pair each receives 0.25 c.c. of 1:100 dilution, and the following ones 0.5 and 1.0 c.c. of the 1:100 dilution; then 0.2, 0.4, 0.8, and 1.6 c.c. of the 1:10 dilution; and finally 0.32 c.c. of the undiluted juice in a total volume of 4 c.c. The remaining pair of tubes contains no juice and serves as a control. Each of the tubes is then covered by the thumb and inverted several times to insure mixing, care being taken to handle the control tubes first, then the pair with the lowest concentration of juice, and so on in order. The rack of tubes is incubated for one hour at 37° C. and is then left in a refrigerator at about 4° C. Twenty to twenty-four hours later the tubes are removed from the refrigerator and read immediately. The lowest dilution in which the gelatin has liquefied in one or both tubes is considered as the reading.

PNEUMONIA, Recognition of Virus Type, Goodrich, B. E., and Bradford, H. A. Am. J. M. Sc. 204: 163, 1942.

Virus-type pneumonia presents specific clinical features permitting diagnosis by positive findings. Diagnosis by exclusion alone is seldom necessary.

Chemotherapy with the sulfonamide group of drugs is not indicated in this disease. Recognition of this disease may avoid needless and possibly dangerous therapeutic efforts.

The initial roentgenographic appearance may closely simulate acute exudative tuberculosis. Progress roentgenographic study may be necessary for differentiation.

There is evidence that this disease is now showing an increasing incidence. This fact is explained not alone by familiarity with the clinical concept.

TUBERCLE BACILLUS, A New Method of Staining, Weiss, E. Am. Rev. Tuberc. 42: 199, 1942.

1. Mordant: The mordant consists of two separate solutions. Solution A contains 100 Gm. of tannic acid dissolved in 100 c.c. of 95 per cent alcohol. Solution B contains 15 c.c. of glacial acetic acid diluted to 100 c.c. with 15 per cent dilution of "solution formaldehyde" (Merck). Before using the mordant, one part of solution A and two parts of solution B are mixed together. This mixture does not require filtering and keeps for several weeks. Solutions A and B keep indefinitely.

2. Safranine (or pyronine) stain: Two and one-half grams of safranine or pyronine are dissolved in 100 c.c. of 10 per cent alcohol. The dissolving process is facilitated by the application of heat. The solutions are then filtered and keep indefinitely.

3. Decolorizer: Fifteen cubic centimeter of glacial acetic acid are diluted to 100 c.c. with acetone. This decolorizer keeps very well.

4. Löffler's methylene blue: Thirty cubic centimeters of a saturated alcoholic solution of methylene blue (5 Gm. dissolved in 100 c.c. of 95 per cent alcohol) are diluted with 100 c.c. of a 1:10,000 solution of potassium hydroxide. The filtrate keeps well.

Staining Technique.—(1) Prepare uniformly thin smears and fix the dry smears over the flame. (2) Cover the slides with the foregoing mordant mixture and steam for five minutes. (3) Wash with running water. (4) Cover the smears with the above safranine or pyronine stain and steam for five minutes. (5) Wash with running water. (6) Decolorize the slides with the above decolorizer until they become colorless. (7) Wash vigorously with running warm water. (8) Cover the smears with Löffler's methylene blue for one minute. (9) Wash with running water and allow the slides to dry. The tubercle bacilli appear as large, thick, deeply red-stained rods while the other bacteria and tissue structures are stained blue.

Evaluation of the procedure: The proposed method has been compared with the Ziehl-Neelsen method on at least 250 diagnostic smears. The tubercle bacilli appeared not only thicker and larger but also more numerous. These observations are based on actual counts performed on the above specimens.

ALKAPTONURIA, The Instantaneous Diagnosis of, on a Single Drop of Urine, Fishberg, E. H. J. A. M. A. 119: 882, 1942.

The urine is made strongly alkaline with sodium or potassium hydroxide and dropped on regulation sensitized photographic paper. Wherever a drop of the urine touches the paper, the latter turns coal black instantly, a process similar to that used in everyday photography. This may be done in full daylight.

No other substance present in urine, either normally or pathologically, is capable of reducing the photographic paper in this manner. Diabetic urine, no matter how high the concentration of reducing sugar, remains without effect. Also, the higher concentrations of vitamin C present in urine after metabolic experiments do not act in this manner.

Since alkaptonuria is either total or absent and the substance is excreted in quantities as high as 16 Gm. a day, usually, however, about 3 to 5 Gm., the reaction is unlikely to be negative in an alkaptonuric patient at any time.

ORAL GLUCOSE TOLERANCE TESTS, Instances of Disagreement in the Results of Two Types of, Langner, P. H., and Dewees, E. J. Am. J. M. Sc. 204: 85, 1942.

From a group of 160 subjects who had submitted to the two-dose, one-hour glucose tolerance test, 16 cases are presented in which the results were border line or slightly abnormal. A one-dose test was also performed on these persons. In 13 instances the one-dose test and the available clinical data indicated a normal carbohydrate metabolism and, therefore, were in disagreement with the two-dose glucose tolerance test. The authors believe that the conventional one-dose glucose tolerance test is more reliable than the newer Exton-Rose procedure.

PORTAL CIRRHOSIS, Gottardo, Paul, and Winters, W. L. Am. J. M. Sc. 204: 205, 1942.

A series of 24 cases of portal cirrhosis of the liver in varying stages was studied in an attempt to correlate clinical and laboratory findings. The diagnosis was confirmed by peritoneoscopy in 20 cases and by post-mortem examination in 4 cases. Marked change in the serum albumin and globulin were noted in 100 per cent of the cases. There was a marked tendency to the lowering of the serum albumin and a rise of the serum globulin, with a consequent lowering of the albumin globulin ratio in all cases. There was a reversal of the albumin-globulin ratio in two-thirds of the cases. Jaundice was a fairly constant feature, being present in some degree, as measured by the icteric index, in all cases. Jaundice was evident on physical examination in 79 per cent of the cases. Changes in the peripheral blood were marked in 96 per cent of the cases. A macrocytic or hyperchromic anemia was present in 67 per cent of the cases while 25 per cent exhibited a microcytic or hypochromic anemia. One case presented a typical severe sickle-cell anemia. Deficiency of liver function, as determined mainly by the hippuric acid test and also by a glucose tolerance and a bromsulfalein test, was noted in 83 per cent of the cases. When the serum protein determinations were utilized as a liver function test, 100 per cent of the cases exhibited a diminution of liver function.

A correlation of clinical and laboratory findings, together with peritoneoscopic examination of the liver and post-mortem analysis, would appear to offer useful fields of investigation in liver disease.

RENAL GLYCOSURIA, The Renal Blood Flow, Glomerular Filtration Rate and Degree of Tubular Reabsorption of Glucose in, Friedman, M., Selzer, A., Sugarman, J., and Sokolow, M. Am. J. M. Sc. 204: 22, 1942.

The effective renal blood flow (diodrast clearance) and the rate of glomerular filtration and the degree of renal tubular reabsorption of glucose were studied in 7 normal persons, and in 5 patients with renal glycosuria.

The effective renal blood flow and glomerular filtration in the patient with renal glycosuria were found to be normal.

The lessened tubular reabsorption of glucose in the person with renal glycosuria does not appear to be due to an organic kidney defect, for at plasma glucose levels above 200 mg. per 100 c.c. the efficiency of tubular reabsorption of glucose in these cases equals or exceeds that found in nonglycosuric patients.

GALACTOSE TOLERANCE, Studies on, With Special Reference to Thyroid Disease, Rosenkrantz, J. A., Bruger, M., and Lockhart, A. J. Am. J. M. Sc. 204: 35, 1942.

The factor of age per se appears to exert little or no influence on the galactose tolerance curve.

Patients with hyperthyroidism, Bright's disease, upper respiratory infections, malignant disease, and those who have recently received sulfonamide therapy frequently demonstrate impairment in galactose tolerance following the oral administration of this sugar. The impairment in galactose tolerance, however, is most marked in patients with thyrotoxicosis, which may be ascribed to a dual mechanism: (a) increased rate of absorption of this sugar from the intestinal tract and (b) hepatic damage.

Patients with diabetes mellitus are shown to have a normal tolerance for ingested galactose.

The Journal of Laboratory and Clinical Medicine

Vol. 28 | November, 1942 | No. 2

PROGRESS

ANTIBODY PRODUCTION AND THE ANAMNESTIC REACTION*

Paul R. Cannon, M.D., Chicago, Ill.

Some important aspects of antibody production have remained obscure for many years because of the doubt concerning the chemical nature of antibodies. The problem of antibody action, therefore, has received more attention than has that of antibody structure. Within the past few years, however, new facts about the chemical constitution of antibodies have suggested new methods of approach to the general problem of antibody formation. The resultant rapidly changing viewpoint can be briefly summarized as follows: In 1929 Wells,[1] in expressing the commonly held opinion of that time, said, "We have absolutely no knowledge what these antibodies may be, or even that they exist as material objects. Like the enzymes, we recognize them by what they do without knowing just what they are. We do not know whether they are specific molecular aggregates or merely physical forces dependent on altered surface energy of the same substances which were already present in the blood before the process of immunization was even begun." Zinsser, two years later, indicated a changing point of view:[2] "It is generally assumed that antibodies cannot be separated from proteins and are usually contained in the pseudoglobulin fraction, in some cases in the euglobulin fractions of the sera." By 1938 Heidelberger had stated the current view in these words:[3] "Although writers on immunology are still to be found who prefer to consider antibodies as ideas, rather than chemical substances, there is now much evidence that antibodies actually are modified serum globulins." And Marrack has recently said (1942):[4] "Now, however, that a large volume of evidence has accumulated which shows that antibodies are modified serum globulins, the chief problem has become the structure and properties of proteins in general and the special characteristics of antibodies in particular." With the chemical composition of antibodies thus definitely established

*From the Department of Pathology, the University of Chicago.

Some of the work on which this discussion is based was aided by the Douglas Smith Foundation for Medical Research of the University of Chicago and the John and Mary R. Markle Foundation.

it should now be possible to proceed more confidently to the study of the conditions that influence their production.

Present-day theories regarding the formation of antibodies all agree in postulating their intracellular production from globulin. The molecules of globulin are synthesized in stereochemical relationship to antigen, and when released, exhibit specific affinities for this antigen. The theory of Breinl and Haurowitz[5] assumes that during the process of antibody formation antigens enter into the mixture of amino acids and peptides and induce new groupings. The formation of antibody thus represents the synthesis of a special kind of globulin in which the molecules orient themselves in ways characteristic for each antigen. As the antibodies pass into the blood the antigen remains behind, thereby continuing to influence the formation of new antibody globulin until the antigenic components are destroyed or eliminated from the antibody-producing cells. Alexander[6] postulates the development within cells of "new specific catalysts" capable of directing antibody synthesis. During their development the antigen becomes an "essential part of the directive surface of the catalyst particles, thereby leading to the formation of groups of particles with reversed electrostatic charge patterns." By such a mechanism minute quantities of antigen, even as little as one molecule, can "convert a cell or an extracellular catalyst into a potential producer of antibody." Mudd's theory[7] embodies the idea that antibodies are synthesized from peptides and amino acids in an "orienting environment, namely, the antigen-protoplasm interface." Amino acids which become coupled to the growing antibody molecule must conform to the "spatial and chemical requirements of the interface in which synthesis is proceeding." Therefore, the antibody corresponds structurally to the configurations of the antigenic surface and hence is specific, i.e., after dissociation it can recombine with antigenic molecules of similar structure. Furthermore, after dissociation the antigen again is available to act as a directive pattern for further synthesis of antibody. Pauling[8] has suggested recently that during antibody formation the molecules of antigen act as directive patterns or templates in the process of globulin synthesis; consequently, a molecule of antibody differs from a molecule of normal globulin only in the portion of the coiled polypeptide chain which has been "templated" during synthesis by contact with portions of the antigen. According to this concept normal globulin and antibody globulin are essentially identical chemically but differ structurally somewhat as one automobile tire may differ from another in tread pattern although being chemically similar. Further verification of this concept is suggested by the recent experiments of Pauling and Campbell,[9, 10] demonstrating the formation of specific antibodies in the test tube by a process of "unfolding" of polypeptide chains, followed by a "refolding" in the presence of antigen. Later dissociation of the complex releases the antibody globulin molecules which can then recombine with their specific antigen.

Inasmuch as these theories and the facts on which they are based all warrant the conclusion that antibody globulin is specifically modified normal globulin, it becomes evident that the mechanism of antibody formation is closely related to that of globulin production. Furthermore, if both types of globulin arise from the same source, deficiencies in the formation of the one must be related to deficiencies in the production of the other. It would seem, therefore, that a clearer understanding of the conditions determining the production of

globulins should help to elucidate both the nature of the antibody mechanism and the processes of antibody production.

Much progress has been made in recent years in the search for the origin of the serum proteins, and many workers have emphasized the importance of the liver; but, as has been pointed out,[11] contradictory experimental results make it impossible to conclude that the liver is the sole source. Reimann, Medes, and Fisher, in their review of the subject,[11] remarked: "It would, however, seem reasonable to search for the site of origin of blood proteins in the blood-forming organs and, for this reason, the bone marrow, the endothelial system including the liver and spleen, and the cellular elements of the blood have been widely studied. Since it is now generally accepted that bilirubin arises from hemoglobin after the dissolution of erythrocytes, it seems plausible to suggest that the plasma proteins might arise in a similar manner from certain cells in the circulation or in the bone marrow, or from tissue cells generally. It would be in the nature of biologic economy to utilize the substances thus liberated, and certain experimental and presumptive evidence supports this view."

Evidence substantiating this suggestion of Reimann, Medes, and Fisher has come both from observation and experiment. Thus Downey and Weidenreich[12] observed the shedding and dissolution of protoplasmic material from lymphocytes and plasma cells and thought that this might indicate a secretory activity of these cells. Sabin[13] reasoned from her earlier studies on the origin of the blood vessels in the chick blastoderm that "since for a vessel to function as such it is essential for the plasma to contain some protein to maintain the balance of intra- and extracellular fluid, the original transformation of a solid mass of angioblasts into a vessel involves the sacrifice of some cytoplasm to make the serum proteins." Her later experiments utilized "marked antigen" of alum-precipitated dye protein, the fate of which, when it was injected into rabbits, could be seen within macrophages. She observed that "coincident with the time when the dye protein is no longer visible within the cells and when there are antibodies in the serum, there is a marked shedding of the surface films of the macrophages without damage to them." If this process of cytoplasmic shedding is a general property of globulin-producing cells, any such cell which can phagocytose antigen or allow its entrance into the cytoplasm may be a precursor of antibody; furthermore, a part of the antibody globulin may remain within the cell for long periods of time instead of being "sacrificed" as blood protein to the surrounding fluids. Landsteiner and Parker[14] have shown, in tissue culture, that in the fluids resulting from the growth of connective tissue cells of chicks in rabbit plasma, proteins are formed which react by precipitin tests with sera from rabbits injected with chicken serum. No attempts were made to determine whether the proteins were albumins or globulins, but the experiments furnished presumptive evidence that connective tissue cells can form serum proteins. It would be of particular interest to know to what extent such cells can produce globulins; their inability to do so adequately and continuously under the artificial conditions of tissue culture might explain the numerous conflicting results of attempts to produce antibodies in vitro.

The idea of the intracellular origin of globulin becomes especially important in connection with the possibility that immune reactions may occur independently of the presence of antibodies in the blood. Besredka,[15] in his formu-

lation of the concept of local immunity, postulated a local tissue immunity without the obligatory participation of antibodies in the circulating fluids. Kahn[16] suggested that specifically immunized tissues may acquire a capacity for union with antigen independent of the presence of antibodies in the blood serum. Seegal and Seegal[17] demonstrated antibodies in ocular tissues in the absence of demonstrable antibodies in the adjacent extracellular fluids. Dienes and Mallory[18] pointed out that hypersensitiveness of the tuberculin type may occur independently of the presence of humoral antibodies and concluded that "if circulating antibodies are involved in the mechanism of this type of hypersensitivity they must be of an unknown nature or else exist in the blood stream in some as yet undetectable form. It appears more probable that we are dealing with an altered tissue reactivity." Walsh and Cannon[19, 20] concluded that regional immunization may lead to the formation or accumulation of antibodies in locally immunized tissues in concentrations higher than in other organs of the same animal and at times even higher than in the blood serum. Although the above workers have all used methods which are admittedly inadequate in some respects, sufficient evidence exists, nevertheless, to indicate the presence of changed reaction capacities of tissues which do not parallel the antibody content of the blood. Inasmuch as the solution of many of the problems of allergy and of tissue immunity depends upon the understanding of the relative influences of cellular and humoral immune reactions, continued efforts must be made to determine their respective potentialities.

An immune phenomenon hitherto not adequately studied is the anamnestic reaction. This phenomenon, while generally recognized as a source of error in the interpretation of certain laboratory procedures, has not received the theoretical attention it deserves. The name "anamnestische serum reaktion" resulted from observations by Conradi and Bieling[21] in 1916 that soldiers previously vaccinated with the typhoid vaccine had a sharp rise in titers in the Gruber-Widal reactions in the course of febrile diseases that stimulated typhoid fever, but that later proved to be something else, as for example, typhus, miliary tuberculosis, pneumonia, erysipelas, bacillary dysentery, Weil's disease, etc. Conradi and Bieling demonstrated in rabbits immunized against typhoid bacilli that the later injection of colon, diphtheria, or dysentery bacilli also caused a sharp rise in the titer of the serum against typhoid bacilli. They concluded, therefore, that the stimulation of antibody-producing cells by heterologous microorganisms caused them to "recall" their previous production of antibodies to typhoid bacilli.

These observations of Conradi and Bieling, although not new, served to emphasize the significance of earlier ones. Dieudonné,[22] for example, as early as 1906 had shown that the injection of sodium cinnamic acid into typhoid-immunized rabbits with low agglutinative titers caused a renewed formation of agglutinins. This demonstration apparently aroused but little interest until the diagnostic problems arising in World War I called renewed attention to the importance of the anamnestic reaction. Thus, Fleckseder[23] in 1916 concluded that many kinds of febrile diseases could cause a sharp rise in the concentration of agglutinins to the typhoid bacillus in the serum of immunized persons, and Weil and Felix[24] saw a rise in titers in the Gruber-Widal reaction in approximately 53 per cent of patients in the course of typhus, as well as to some degree

in other infectious processes. In 1917 Dreyer and Inman[25] emphasized the importance of quantitative methods in agglutination tests in typhoid-immunized persons with enteric disease because of the concomitant production of major and minor agglutinins. In the same year Baehr[26] said: "It has been occasionally noted that the Widal reaction may become positive during any febrile disorder in persons who previously had typhoid fever or who have recently been vaccinated with typhoid bacilli. . . This temporary lighting up of the Widal reaction during typhus fever was commonly observed by the German and Austro-Hungarian army bacteriologists in Russian Poland and Volhynia, for all the troops had received typhoid vaccinations." It was not known until many years later that the capacity to produce agglutinins to the typhoid bacillus after immunization may persist in healthy persons for as long as from six to nine years.[27]

The demonstration by Dieudonné that nonspecific agents can elicit the anamnestic response was confirmed in 1917 by Kirstein[28] in his experiments showing that infection of typhoid-immunized rabbits with vaccinia virus also caused a mobilization of agglutinins to the typhoid bacillus. He emphasized particularly the fact that nonbacterial agents had a stimulative effect. In all of these earlier observations, however, the primary emphasis was on the practical aspects of the reaction because of the diagnostic difficulties presented, and only a few efforts were made to ascertain the cause of the anamnesis. Some attributed the reaction to the reappearance of "preformed antibodies";[26] others thought that the acute hyperplasia of the spleen was responsible; only Conradi and Bieling made any attempt to determine experimentally the underlying nature of the phenomenon.

The anamnestic phenomenon can be demonstrated, also, against nonbacterial antigens. Obermeyer and Pick[29] in 1904 observed the reappearance of precipitins to beef serum after injection of horse albumoses in rabbits previously injected with beef serum. Little attention was paid to this aspect, however, until 1917, when Hektoen[30] pointed out an important source of error in the production of precipitins for foreign sera in that, when rabbits were injected with one type of serum and were later injected with a heterologous serum, precipitins to the first serum reappeared. He said at that time: "The particular point on which special stress is placed now is the capacity of the rabbit under suitable conditions . . . to elaborate different precipitins at the same time. It appears that the precipitin production induced in the usual way leaves behind it an increased power of further production so that large amounts of major as well as group and minor precipitins are elaborated on the injection of a new serum or blood. Manifestly, the phenomenon is an expression of an increased reactivity and may be classed with other manifestations of allergy." Bieling,[31] in later experiments, re-emphasized the idea that the changed reaction capacity of tissues is an expression of allergy whereby the power of a cell to produce antibody can be changed both specifically and *nonspecifically*. He visualized the immune state in a cell as a process of development of a nonspecific hypersusceptibility to other kinds of stimuli: as a result of this, later stimuli can engender the appearance of nonspecific antibodies in the pretreated animal. Mackenzie and Frühbauer[32] reported that after precipitins to foreign proteins had disappeared from the blood, later injection of another protein caused them to

reappear; furthermore, the precipitins to the last antigen previously injected were the first to reappear. Hektoen and Welker[33] have shown, also, that subsequent to the induction and disappearance of multiple precipitin formation by the successive introduction of *different* antigens, the injection of only one of the antigens previously used may cause the reappearance of precipitins to the same as well as the other antigens.

During the entire period of study of the anamnestic reaction, unnecessary confusion has centered around the use of the term itself. The word "anamnesis" means "a recalling," and in the anamnestic reaction the antibody-producing cells presumably "recall" or "remember" a previous antigenic experience during the course of a subsequent and frequently unrelated type of cellular stimulation. Because of the heterologous nature of the later stimulus too sharp a restriction has been placed upon this feature of the phenomenon. As Topley[34] has said: "In this connection we note the terminological confusion which has sometimes arisen in the literature. The production, in response to an antigenic stimulus, of an antibody that has been produced in the tissues on some previous occasion is often referred to as an anamnestic reaction (anamnesis recollection). Sometimes this term is used in connection with the type of nonspecific stimulus to which we have just referred—the tissues are stimulated to general activity, in which is included the renewed production of a particular antibody that has been formed at some time in the past. Sometimes, perhaps more accurately, it is employed in reference only to those instances in which the particular chemical stimulus concerned formed part of the earlier experience of the antibody-producing cells." There can be no doubt, therefore, that Topley applies the term "anamnestic reaction" both to the nonspecific and the specific liberation of antibodies into the blood stream consequent to a later antigenic stimulation. This broader point of view implies, therefore, that if antibody-producing cells are to manifest some type of "recollection" of an earlier antigenic experience, there is no reason why this should happen only after a nonspecific stimulation. The determining feature of the anamnestic reaction is the speedy release of antibodies from their site of production, regardless of the stimulus which may bring it about. Acceptance of this point of view makes it immaterial whether the reaction is called "anamnestic," "secondary immune response," "injection de rappel," or the reaction to "stimulating," "booster," or "repeat" injections.

In the present discussion the anamnestic reaction is considered to be the result either of nonspecific or specific stimulation of antibody-producing cells which have undergone earlier antigenic stimulation. After nonspecific stimulation there is the reappearance of antibodies to the earlier antigen; after specific stimulation there is an earlier and more abundant reappearance of antibodies to the specific antigenic stimulant.

The importance of the anamnestic phenomenon is seen also in infectious processes. For example, the use which is now being made of the so-called "stimulating" or "booster" injections of toxoids in order to call forth an accelerated liberation of antitoxins[35-38] is an instance of "specific anamnesis," whereby higher levels of antitoxic immunity are attained in children and in soldiers who have been immunized previously with toxoids of diphtheria and tetanus. Jones and Moss describe the case of a person who eight months after immunization with tetanus toxoid suffered a puncture wound of the hand.

His blood serum, when tested after immunization, had shown the following antitoxic levels: at six weeks, one-tenth unit per cubic centimeter; at twelve weeks, one-twentieth unit; at six months, one-fortieth unit. On the day following the injury he was reinjected with 0.25 c.c. of alum-precipitated tetanus toxoid; his blood serum, tested one week later, contained one-tenth unit per cubic centimeter, and four weeks later, one-half unit. The authors estimated that his total blood, therefore, would have contained approximately 3,000 units of antitoxin, or "more than enough antitoxin to protect against the development of clinical tetanus" as well as to possess an active immunity far superior to the passive protection which would have been possible by the injection of antitoxin. These authors showed later, moreover, that stimulating injections of toxoid not only accomplished the accelerated elevation of antitoxic levels in the blood, but that these levels persisted longer than after first injections. It is not unlikely that in persons adequately immunized with toxoids a later infection with diphtheria or tetanus bacilli will, because of the speedy liberation of small quantities of toxin into the tissues, engender an accelerated secondary response and thus bring freshly liberated antitoxins quickly to the site of developing infection, thereby favoring an early termination of the infective focus.

The anamnestic reaction may operate similarly in infections in which antigenic stimulations accompanying the acute infectious process may liberate, in varying degrees, antibodies engendered by earlier antigenic experiences. It is conceivable that such a response would help materially in restraining the development of incipient secondary infections which might otherwise complicate seriously the major infectious process. For example, Hektoen and Boor[39] called attention to the fact that "the serum of apparently healthy persons contains, as is well known, small quantities of a great variety of antibodies for infectious, as well as non-infectious, antigens . . . it seems reasonable to assume that 'normal antibodies' may develop in response to antigens that enter the blood from the digestive and respiratory tracts, as well as in other ways."

It may be recalled that during the first World War the most serious complications of influenza and measles occurred in soldiers from the sparsely settled rural areas. These men, presumably, had had fewer antigenic experiences with microorganisms commonly implicated in respiratory infections than had soldiers coming from the thickly populated urban areas. It is possible that, during the course of influenza or measles, the anamnestic mobilization of multiple antibodies to various types of streptococci and pneumococci may have played an important part in restricting the early growth and spread of secondary bacterial invaders which might otherwise have caused serious or even lethal infections.

The anamnestic reaction may also play a beneficial role in the treatment of chronic or focal infections in which, presumably, foci of infective agents persist in the tissues over long periods of time. For example, in the treatment of chronic syphilis, arthritis, or iritis, stimulation of the antibody mechanism by various foreign protein materials, such as typhoid vaccine, milk, malarial parasites, or by hyperthermic means, may cause the liberation into the blood stream of significant amounts of specific antibodies engendered by previous antigenic experiences but which could not otherwise gain access to the infected foci. According to Cecil,[40] "perhaps the most important function of the foreign protein reaction is the mobilization of immune bodies in the circulating blood. . . . Indeed, it has

been shown by numerous authors that any form of fever is conducive to an increase of immune bodies in the circulating blood. Whether this is due to an increased formation or to increased mobilization of antibodies it is difficult to say." O'Leary[41] also remarks: "The literature contains numerous hypotheses as to the mechanism that produces the satisfactory results of fever therapy, but not one of these offers sufficient evidence to warrant its acceptance except as a theory. In the fever induced by the malarial treatment, as well as in that produced by other nonspecific agents, it is my impression that the satisfactory therapeutic effects are the result of some fundamental change in the immunologic processes, the nature of which is unfamiliar. The high temperatures which are produced may be a factor in bringing about these changes." Although more examples might be cited, these will suffice to illustrate the general importance of the phenomenon, not only through its effects upon various laboratory procedures, but also upon many important problems of acquired resistance.

Perhaps, however, the relationship of the anamnestic reaction to the problem of antibody production is of even greater significance. Although it is now generally agreed that the antibody-producing mechanism consists predominantly of macrophages comprising the reticulo-endothelial system, it is possible that antibody formation may be more widespread. In fact, any cell which can actively phagocytose foreign antigenic materials, or even allow them to enter its cytoplasm and remain there while globulin is being synthesized may, presumably, either produce antibody for release into the circulating fluids or develop a changed reaction capacity to the same type of antigen. It seems probable, therefore, that several kinds of cells may play an important part in the synthesis of globulin and that tissue globulin functions as the mother substance or matrix from which antibodies are formed, regardless of whether they are liberated into the blood or remain at their sites of formation.

Such a matrix of globulin, if it is to function effectively both for the production of antibodies and for the preservation of the antibody mechanism itself, must be adequate qualitatively and quantitatively. Consequently, the reserves of tissue globulin must have been properly constructed during synthesis, and in sufficient amounts. Inasmuch as these arise ultimately from amino acids in the food, nutritional conditions suitable for the effective synthesis of globulin are necessary. This particular aspect of the problem will not be discussed in the present paper, since it has been considered in more detail elsewhere.[42] It is a well-known fact, however, that the production of antibodies is, as a rule, poorer in conditions of immaturity, severe malnutrition, and excessive loss of blood proteins. While the causes of this impaired capacity to form antibodies are still unknown, antibody-producing cells possessing an adequate content of tissue globulin of good quality should be capable of synthesizing as many varieties of specific antibodies as there are antigenic templates to influence their specific formation. Hektoen and Boor[39] have shown that the injection of 35 different antigens at one time engendered the simultaneous formation of 34 specific antibodies. Furthermore, Delves[43] found that the immunization of rabbits with a mixture of ten kinds of laked blood led to the simultaneous production of specific precipitins for many blood proteins and specific agglutinins and lysins for different species of blood corpuscles. These findings indicate that, under normal conditions of antibody formation, antibody synthesis depends upon

the presence within the antibody-producing cells of antigenic components, regardless of their numbers at any one time. The process may be likened to that of the printing of letters of dough for the preparation of "alphabet soup." If one compares the dough to tissue globulin, one can think of it as passing through a stamping machine with the various letters of the alphabet emerging. If, however, some of the letter molds become injured or lost, the letters will come out poorly marked or not marked at all. Furthermore, if the supply of dough runs low or deteriorates in quality because of dilution or other change, letters of poor quality will emerge.

If globulins, whether normal or specifically modified by antigen are products of cells, it becomes essential that the supply of amino acids for proper cellular functioning be constantly maintained. Furthermore, it may be highly important that the intracellular reserves of protein materials be maintained in these or in other cells of the body in order that globulin synthesis may go on uninterruptedly and even at an accelerated tempo in the presence of infection. Whipple and his collaborators[44] have shown that protein materials, particularly in the liver, constitute an important reservoir of readily mobilizable protein aggregates which are in dynamic equilibrium with the blood proteins and which can be called upon in time of need for the nourishment of the protein-depleted tissues. Schoenheimer and his associates[45] have shown also that the process of interchange of amino acids within the tissues is extremely dynamic, and that, if during the process of antibody synthesis, an amino acid (glycine) containing isotopic nitrogen is fed to a rabbit, some of the heavy nitrogen quickly appears in the antibody globulin itself. It is evident, therefore, that even during the period of antibody formation amino acids in the food may play an active part in the construction of antibody molecules.

This evidence of the active participation of amino acids of the food in antibody production re-emphasizes the problem of protein metabolism during infection. The loss of body protein during infectious disease, due to the fever, the toxemia, and the insufficient regeneration because of inadequate food intake, may be an important factor in the depletion of the protein reserves. For example, patients with typhoid fever may lose as much as 100 Gm. of body nitrogen in from eight to twelve days; the outstanding clinical fact in such patients is starvation. One may wonder in retrospect about the consequences of the low-calorie diets in the treatment of typhoid fever, particularly during those stages of the disease in which the tissues, presumably, needed an adequate supply of immune substances. It may be that the not infrequent relapses of those times were caused, in part, by the scarcity of amino acids essential for the continued production of antibodies; and perhaps the favorable effects of the Coleman-Shaffer high-calorie diet,[46] with its correspondingly higher content of amino acids, were due in some degree, to the better production of antibodies as well as to the greater calorie values and the protein-sparing effects of the carbohydrate. Coleman[47] has said concerning prognosis in typhoid fever that "when a patient is well nourished, the natural history of the disease is profoundly modified in many respects. There is evidence that complications occur less frequently. Such complications as do occur are less serious in their consequences. The immunity processes appear to be favorably influenced." He states, furthermore, that from his observations "the mortality rates in patients who were well

nourished were from 50 per cent to 75 per cent lower than in those who were partially starved." These observations suggest that in other subacute and chronic infectious diseases, as for example, tuberculosis, syphilis, malaria, rheumatic disease, etc., good nutrition may also facilitate recovery to a considerable degree by its action in promoting the more effective synthesis of antibody globulin.

The probability that the antibody mechanism is affected by starvation is of particular importance today as one of the problems of war. The association of war, famine, and pestilence has long been known, but the reasons for the rise of epidemics and the higher death rates from infection are still not well understood. During World War I the incidence of tuberculosis rose greatly in Germany and elsewhere, and it has been estimated that at least 800,000 persons died as a result of the food blockade, many because of infectious diseases. Part of this high death rate has been attributed to the lowered intake of protein in the diet. The possibility that depleted protein reserves may reduce the capacity of a starving person to produce antibody-globulin quickly and effectively may be of profound importance in any incipient infection which might otherwise be readily terminated. Experiments now in progress indicate very definitely that rabbits with hypoproteinemia, the result of plasmapheresis and a low protein diet, show an impaired ability to form antibodies. Such experiments suggest the tremendous importance of maintaining adequate protein reserves; the only way, obviously, is by the ingestion of sufficient amounts of protein in the diet.

The question whether there are reserves of stored protein in the tissues comparable to those of glycogen and fat is controversial, although there is but little doubt that under some conditions, as after hemorrhage or plasmapheresis and during other hypoproteinemic states, organs and tissues may contribute proteins to the blood. Nor is it known in what form or where the protein reserves are stored, although the liver undoubtedly plays an important and perhaps a predominant role. Berg[48] claimed many years ago that in well-nourished animals protein deposits could be demonstrated microscopically in the liver cells as intracellular "protein inclusions"; in starving animals these inclusions disappeared and the cells became smaller. Furthermore, Berg and Cahn-Bronner[49] showed that, when starved animals (salamanders and rabbits) were fed a mixture of amino acids obtained from enzymatic hydrolysis of beef flesh, these inclusions reappeared in the liver cells within a few hours and were indistinguishable from those seen after ordinary protein feeding. They concluded, therefore, that these represented a phase of protein storage. Elman and Heifetz[50] showed, furthermore, in dogs kept on a low-protein diet, that as the albumin values of the blood fell, there was a concomitant decrease in the amount of stainable cytoplasm in the hepatic cells. The latter became more vacuolated and the water content of the liver as a whole increased.

There is no conclusive evidence as yet, however, as to the chemical state in which protein is stored in the liver. Luck[51] maintained two groups of rats on high- and low-protein diets, respectively, and analyzed the livers quantitatively for evidence of protein storage. He found that in the high-protein group there was an increased amount of protein per unit weight of tissue but that all the protein fractions were increased about proportionally when compared with the

similar fractions in the low-protein group. Addis and his associates,[52] in quantitative studies on the effects of fasting in rats upon protein storage, found that the liver in seven days lost about 40 per cent of its total protein, thus indicating, also, the importance of this organ as a storehouse or way station in protein metabolism. Whipple and his collaborators have demonstrated by physiologic methods the important part played by stored proteins in the bodily economy as a "reserve against adversity in the sense that it ean be circumspectly depleted without apparent injury to the body." Whipple suggests that intracellular proteins may exist in three forms, viz., labile reserve protein, dispensable reserve protein, and indispensable fixed protein, and that, because of the existence of a dynamic equilibrium between the fluids and the tissues, there is an "easy exchange between the plasma protein and the labile protein stores of the body cells." Under conditions of anemia, plasma depletion, or protein fasting even the dispensable reserve proteins may also be drawn upon to some extent. The indispensable fixed proteins, however, cannot be removed unless the cells are seriously damaged or destroyed. It is of interest, furthermore, that when the "stored" proteins are contributed to the blood stream under the above conditions, the most labile protein reserves seem to be those with larger molecules. Thus Berger[53] has shown that after the injection of a foreign protein into rabbits, the resulting hyperproteinemia occurs primarily (in about four days) as a hyperglobulinemia with an associated hypoalbuminemia. The albumin levels rise later, usually in from sixty to one hundred and twenty days. Similarly, after severe blood loss in normal animals there is an increase in normal globulin in the blood, whereas, in immnnized ones antibody globulin is quickly liberated into the blood, as seen in the anamnestic response after severe hemorrhage.

The facts cited in this brief review of the problem of antibody production and the anamnestic reaction suggest that antigenic components may persist within antibody-producing cells for considerable periods of time after injection or vaccination and thus are able to function as templates when later stimuli cause an acceleration of antibody prodnetion. Furthermore, there is evidence that antigenic templates which were effective earlier in the process of antibody formation tend to disappear or to become changed with time so that they lose their ability to influence specifically the later synthesis of globulin. Further studies of the anamnestic phenomenon should throw more light upon many more important aspects of the general problem of antibody production.

SUMMARY AND CONCLUSIONS

The antibody-producing mechanism comprises a widely distributed system of cells, including macrophages, fibroblasts, and possibly even endothelial cells and to some extent epidermal cells, all of which may have the potentiality in varying degrees of synthesizing globulin. Wherever this material is formed, antigenic "templates" may, if present at the sites of synthesis, so influence the structural pattern of the globulin molecules as to confer specific properties upon them. Some of these molecules may pass into the extracellular fluids as antibodies and remain there for variable periods of time; others may persist within their cells of formation. The persistence of this antibody matrix for months or years may explain both the acquisition of acquired resistance inde-

pendently of any considerable amounts of antibodies in the blood as well as many of the tissue reactivities which are commonly regarded as allergic phenomena. The effectiveness of globulin synthesis, and consequently, of antibody production will be determined by the quality and quantity of globulin reserves continuously available. These, in turn, are dependent ultimately upon an adequate intake of amino acids in the food. Diet, therefore, will determine to a large degree both the effective production of antibody globulin and the potential reserve capacity of the antibody mechanism.

REFERENCES

1. Wells, H. Gideon: The Chemical Aspects of Immunity, New York, 1929, The Chemical Catalog Company, page 109.
2. Zinsser, Hans: Resistance to Infectious Disease, ed. 4, New York, 1931, The Macmillan Company, page 133.
3. Heidelberger, Michael: The Chemistry of the Amino Acids, Carl L. A. Schmidt, Editor, Springfield, 1938, Charles C Thomas Company, Relation of Proteins to Immunity, page 959.
4. Marrack, John R.: Immunochemistry, Ann. Rev. Biochem. **11:** 629, 1942.
5. Breinl, Friedrich, and Haurowitz, Felix: Chemische Untersuchung des Präzipitates aus Hämoglobin und Anti-hämoglobin-Serum und Bemerkungen über die Natur der Antikörper, Ztschr. f. physiol. Chem. **181:** 45, 1930.
6. Alexander, Jerome: Some Intracellular Aspects of Life and Disease, Protoplasma **14:** 296, 1931.
7. Mudd, Stuart: A Hypothetical Mechanism of Antibody Formation, J. Immunol. **23:** 423, 1932.
8. Pauling, Linus: Theory of Structure and Process of Formation of Antibodies, J. Am. Chem. Soc. **62:** 2643, 1940.
9. Pauling, Linus, and Campbell, Dan H.: The Production of Antibodies in Vitro, Science **95:** 440, 1942.
10. Pauling, Linus, and Campbell, Dan H.: The Manufacture of Antibodies in Vitro, J. Exper. Med. **76:** 211, 1942.
11. Reimann, Hobart A., Medes, Grace, and Fisher, Luther: The Origin of Blood Proteins, Folia haemat. **52:** 187, 1934.
12. Downey, Hal, and Weidenreich, Franz: Ueber die Bildung der Lymphocyten in Lymphdrüsen und Milz, Arch. f. mikr. Anat. **80:** 306, 1912.
13. Sabin, Florence R.: Cellular Reactions to a Dye-Protein With a Concept of the Mechanism of Antibody Formation, J. Exper. Med. **70:** 67, 1939.
14. Landsteiner, Karl, and Parker, Raymond C.: Serological Tests for Homologous Serum Proteins in Tissue Cultures Maintained on a Foreign Medium, J. Exper. Med. **71:** 231, 1940.
15. Besredka, A.: Local Immunization, Baltimore, 1927, Williams & Wilkins Company.
16. Kahn, Reuben L.: Tissue Reactions in Immunity. XIV. The Specific Reacting Capacities of Different Tissues of an Immunized Animal, Science **79:** 172, 1934.
17. Seegal, Beatrice C., and Seegal, David: Local Organ Hypersensitiveness. VII. Demonstration of Agglutinins in Tissues of the Rabbit Eye Following Immunization With E. typhi Vaccine, Proc. Soc. Exper. Biol. & Med. **31:** 437, 1934.
18. Dienes, Louis, and Mallory, Tracy B.: Histological Studies of Hypersensitive Reactions, Am. J. Path. **8:** 689, 1932.
19. Walsh, Theodore E., and Cannon, Paul R.: Immunization of the Upper Respiratory Tract, Arch. Otolaryngol. **20:** 820, 1934.
20. Walsh, Theodore E., and Cannon, Paul R.: Immunization of the Respiratory Tract: A Comparative Study of the Antibody Content of the Respiratory and Other Tissues Following Active, Passive and Regional Immunization, J. Immunol. **35:** 31, 1938.
21. Conradi, H., and Bieling, R.: Ueber Fehlerquellen der Gruber-Widalschen Reaktion, Deutsche med. Wchnschr. **42:** 1280, 1916.
22. Dieudonné, A.: Steigerung der Agglutininbildung durch nicht-spezifische Stoffe, Med. Klin. **2:** 575, 1906.
23. Fleckseder, Rudolf: Ausschwemmung von Typhus agglutinin durch Fieber verschiedener Herkunft, Wien. klin. Wchnschr. **29:** 637, 1916.
24. Weil, E., and Felix, A.: Ueber die Beziehungen der Gruber-Widalschen Reaktion zum Fleckfieber, Wien. klin. Wchnschr. **29:** 974, 1916.
25. Dreyer, Georges, and Inman, A. C.: The Agglutination Curve and Its Importance in the Diagnosis of Typhoid and Paratyphoid Fevers in Inoculated Persons, Lancet **1:** 365, 1917.
26. Baehr, George: Agglutination in Typhus Fever, J. Infect. Dis. **21:** 21, 1917.

27. Foord, Alvin G., and Forsyth, Anna: The Agglutinin Content of the Blood Following Typhoid and Paratyphoid Immunization, Am. J. Clin. Path. 3: 333, 1932.
28. Kirstein, Fritz: Steigerung der Gruber-Widalschen Reaktion durch Vakzinevirus, Deutsch med. Wchnschr. 43: 325, 1917.
29. Obermeyer, E., and Pick, E. P.: Beiträge zur Kenntnis der Präzipitinbildung, Wien. klin. Wchnschr. 17: 265, 1904.
30. Hektoen, L.: Precipitin-Production in Allergic Rabbits, J. Infect. Dis. 21: 279, 1917.
31. Bieling, R.: Untersuchungen Ueber die veränderte Agglutininbildung mit Ruhrbacillen vorbehandelter Kaninchen, Ztschr. f. Immunitätsforsch. u. exper. Therap. 28: 246, 1919.
32. Mackenzie, G. M., and Frühbauer, Emily: The Anamnestic Reaction: Response of Previously Immunized Animals to Heterologous Antigens, Proc. Soc. Exper. Biol. & Med. 24: 419, 1927.
33. Hektoen, Ludvig, and Welker, William H.: Renewal of Multiple Precipitin Production on Injection of One Antigen in Rabbits Successively Immunized With Many Antigens, Science 86: 592, 1937.
34. Topley, W. W. C.: An Outline of Immunity, Baltimore, 1933, William Wood and Company, page 183.
35. Bigler, John A., and Werner, Marie: Active Immunization Against Tetanus and Diphtheria in Infants and Children, J. A. M. A. 116: 2355, 1941.
36. Jones, F. G., and Moss, James M.: The Antitoxic Titers of Human Subjects Following Immunization With Combined Diphtheria and Tetanus Toxoids, Alum Precipitated, J. Immunol. 33: 173, 1937.
37. Jones, F. G., and Moss, James M.: Studies on Tetanus Toxoid. II. The Response of Human Subjects to an Injection of Tetanus Toxoid or Tetanus Alum Precipitated Toxoid One Year After Immunization, J. Immunol. 33: 183, 1937.
38. Jones, F. G., and Moss, James M.: Combined Diphtheria and Tetanus Toxoid, Alum Precipitated, J. Lab. & Clin. Med. 24: 512, 1939.
39. Hektoen, Ludvig, and Boor, Alden K.: Simultaneous Multiple Immunization, J. Infect. Dis. 48: 588, 1931.
40. Cecil, Russell L.: Nonspecific Protein Therapy, J. A. M. A. 105: 1846, 1935.
41. O'Leary, Paul A.: Nonspecific Treatment of Syphilis, J. A. M. A. 110: 42, 1938.
42. Cannon, Paul R.: Antibodies and the Protein Reserves, J. Immunol. 44: 107, 1942.
43. Delves, Edna: Simultaneous Immunization With a Mixture of Ten Kinds of Laked Blood, J. Infect. Dis. 57: 61, 1935.
44. Madden, Sidney C., and Whipple, George H.: Plasma Proteins: Their Source, Production and Utilization, Physiol. Rev. 20: 194, 1940.
45. Schoenheimer, Rudolf: The Dynamic State of Body Constituents, Cambridge, Mass., 1942, Harvard University Press.
46. Shaffer, Philip A., and Coleman, Warren: Protein Metabolism in Typhoid Fever, Arch. Int. Med. 4: 538, 1909.
47. Coleman, Warren: Typhoid Fever, Nelson Loose-Leaf Living Medicine, New York, 1920, Thomas Nelson and Sons, Vol. 1, page 175.
48. Berg, W.: Ueber den mikroskopischen Nachweiss der Eiweiszspeicherung in der Leber, Biochem. Ztschr. 61: 428, 1914.
49. Berg, W., and Cahn-Bronner, C.: Ueber den mikroskopischen Nachweiss der Eiweiszspeicherung in der Leber nach Verfütterung von Aminosäuren, Biochem. Ztschr. 61: 434, 1914.
50. Elman, Robert, and Heifetz, Carl L.: Experimental Hypoalbuminemia. Its Effect on the Morphology, Function and Protein and Water Content of the Liver, J. Exper. Med. 73: 417, 1941.
51. Luck, James Murray: Liver Proteins. I. The Question of Protein Storage, J. Biol. Chem. 115: 491, 1936.
52. Addis, T., Poo, L. J., and Lew, W.: The Quantities of Protein Lost by the Various Organs and Tissues of the Body During a Fast, J. Biol. Chem. 115: 111, 1936.
53. Berger, Wilhelm: Ueber die Hyperproteinämie nach Eiweiszinjektionen. Ein experimenteller Beitrag zur Pathologie des Serumproteins und sur Proteinkörpertherapie, Ztschr. f. d. ges. exper. Med. 28: 1, 1922.

CLINICAL AND EXPERIMENTAL

SARCOIDOSIS (BOECK-BESNIER-SCHAUMANN DISEASE) AS CAUSE OF A PITUITARY SYNDROME*

E. J. Kraus, M.D., Peoria, Ill.

COMPARED with other anatomic lesions, the involvement of the pituitary gland by sarcoidosis represents a rare event. According to a recent review on this disease by Longcope,[1] only six cases of pituitary involvement seem to have been published, all accompanied by diabetes insipidus.

The case represented in this paper differs from previous cases in that the pituitary region showed particular findings which might not have been described yet in this combination.

Today we know that sarcoidosis or Boeck's sarcoid is identical with lupus pernio described by Besnier, and that uveoparotid fever represents only a particular manifestation of the same disease. It is a chronic disease of long duration, usually characterized by a mild, often feverless, course, a tendency to exacerbation and refractory behavior to treatment, and often shows spontaneous recession. The underlying process is a granuloma with a certain resemblance to that of tuberculosis. Sarcoidosis is not identical with tuberculosis, but is often combined with it. The most frequent organs attacked are the lungs, lymph nodes, skin, spleen, liver, and eye; however, not many organs escape being involved by the granuloma. Sarcoidosis with especial involvement of the skeleton has been described erroneously by Jüngling,[2] a German author, as osteitis tuberculosa cystica multiplex.

The granulation tissue in sarcoidosis has no tendency to undergo caseation and is characterized by nodules consisting of epithelioid cells, lymphocytes, and giant cells with peculiar calcified inclusions. These nodules are frequently surrounded by a wall of hyalinized fibrous tissue. They are not prone to coalesce but remain separate though often closely approximated. The etiology of the disease is not known; transmission to animals has not been successful so far. Among the laboratory findings, one of the most significant is hyperglobulinemia.

CASE REPORT

The following case is that of a woman, aged 41 years, a teacher by occupation. Her menstruation began at 15 years of age and continued regularly for two years, the periods lasting three days. Then the menses disappeared without discernible cause, after which she bled from the nose quite regularly for two or three days every month. Prior to her death the epistaxis was so heavy that it had to be stopped at the hospital.

*From the Department of Pathology at St. Francis Hospital, Peoria.

Received for publication, May 20, 1942.

The patient gave a history of previous apical tuberculosis. She was anemic for about ten years. She died shortly after admission, her temperature having risen to 104° F.

The clinical diagnosis was hypochromic anemia, thrombocytopenia, hypoplasia of the genitals, and polyuria.

Fig. 1.—Sagittal section through the skull base, showing the dorsum sellae strikingly high and prominent.

Fig. 2.—Aspect of the cranial base, showing the atrophic pituitary gland lying deep in the sella with the flattened pituitary stalk running over the abnormally high dorsum sellae.

Autopsy.—The body was 149 cm. in length and 46 kg. in weight, proportional in its dimensions. Panniculus adiposus measured 3 cm. over the abdomen, 1 cm. over the external bicipital sulcus, and 3 cm. over the fossa ovalis. The skin was without particular pigmentation. The head hair was brown and thinner than normal; the axillae, were without hair; the pubic hair was blonde, scanty, and downy; and the trunk and extremities were hairless. The breasts were small, flat, and poor in fat tissue; the nipples were not prominent; labia majora and labia minora were small.

The skull measured 17 by 14.5 cm. in diameter and up to 0.4 cm. in thickness, with a circumference of 50.5 cm. Brain and meninges were grossly without particular lesions. The pituitary gland on its cranial surface was excavated and lay deep in the sella; the dorsum sellae was unusually high and bent slightly forward. The pituitary stalk which passed over the prominent edge of the dorsum sellae was lengthened, flattened, and whitish in color (Figs. 1 and 2).

The nasal cavity was filled with coagulated blood. The thyroid was small and atrophic. In the left upper lobe of the lungs there was a deep transverse scar with neighboring bronchiectasis. The left apex contained a slaty indurated area, about 2 cm. in diameter, and two smaller calcified foci. There were fibrous adhesions of the apical pleura. Both lungs were

firmer than normal and had reduced air content; the surface in many parts was rough and showed numerous tiny grayish nodules closely compacted. In the left lower lobe a fine network of scar tissue was found in some places. The lobes of both lungs were fused together by fibrous adhesions. In other parts the pleura showed ecchymoses, as did the epicardium. The myocardium, the heart valves, and the aorta did not reveal essential changes. The bronchopulmonary and tracheobronchial lymph nodes were indurated, anthracotic, and contained a few calcified foci. The mediastinal, abdominal, and retroperitoneal lymph nodes were enlarged, especially the perigastric, peripancreatic, and para-aortal ones, which appeared to be about 2 by 3 cm. in diameter, while the iliac, hypogastric, internal inguinal, and mesenteric lymph nodes were only the size of a bean. They were firm in consistency and permeated by grayish-white nodules the size of millet seeds, the nodules being closely approximated in many places.

The spleen weighed 500 Gm., showed fibrous adhesions, and on its cut surface were many miliary nodules almost indistinguishable from the follicles. The liver, partially covered by fibrous adhesions and weighing 1,700 Gm., was light yellowish-brown in color and indurated, offering the picture of a moderate degree of cirrhosis. The gall bladder was shrunken and contained a cholesterol-pigment calcium stone. The pancreas appeared to be normal. The adrenals were small. The kidneys together weighed 200 Gm. They were firm and light yellow in color.

Fig. 3.—One nodule after decalcification, showing foreign body giant cells surrounding the site of the inclusions.

The uterus was small, measuring 5 by 3 cm. The corpus wall measured up to 0.5 cm. in thickness. The tubes were 11 and 12 cm., respectively, in length and very thin. The ovaries were small and shrunken, the vagina was narrow, measuring 6.5 cm. in circumference.

The femurs, ribs, sternum, and vertebras showed signs of osteoporosis. Two-thirds of the marrow in the femur consisted of light gray-red myeloid tissue while one-third was made up of fat tissue.

Anatomical Diagnosis.—Abnormal height of the dorsum sellae with compression, extension, and atrophy of the pituitary stalk; atrophy of the pituitary gland; hypotrichosis; atrophy of the ovaries, uterus, tubes, and labia pudenda; atrophy of the breasts; atrophy of thyroid and adrenals (hypopituitarism); old tuberculosis of the lung apices with fibrous adhesive pleuritis; circumscribed bronchiectasis of the left lung; peculiar granuloma (?) in all lobes of the lungs, all internal lymph nodes (apparently) and spleen; chronic splenic tumor; cirrhosis of the liver; chronic cholecystitis and cholelithiasis; osteoporosis; hemorrhage in the nasal cavity; ecchymoses in serous membranes; anemia.

Bacteriologic Examination.—Cultures on Löwenstein medium taken from spleen, lymph nodes, and bone marrow for tubercle bacilli were sterile after six weeks' observation. Guinea pigs, rabbits, and chickens inoculated with the same tissues did not show any lesions after days. Guinea pigs inoculated with lymph nodes, spleen, liver, and lung of these 'oned animals showed no pathologic lesions after fifty-three days.

Microscopic Examination.—Lungs. There are numerous miliary nodules of variable appearance. Some consist of epithelioid cells, one or a few giant cells similar to Langhans giant cells, and some round cells; frequently these are all intermingled. Other nodules are larger and contain dark violet inclusions (stained by hematoxylin), spindle, round, or rod shaped. Other nodules contain bodies suggestive of cholesterol crystals. The giant cells are intimately attached to these structures, as is typical of foreign body giant cells (Fig. 3). A number of nodules are surrounded by a thin wall of hyalinized fibrous tissue, while others appear to be fibrous also in the center. There is no tendency to conglomerate, though the nodules frequently appear close together. The final stage is a hyalinized scar containing recent nodules as well as calcified inclusions. These inclusions, which chiefly consist of calcium carbonate and calcium phosphate, also give a positive iron reaction, as shown in Turnbull blue sections. In some parts the alveolar septa are thickened, infiltrated with many lymphocytes, and contain recent as well as older and partially hyalinized nodules.

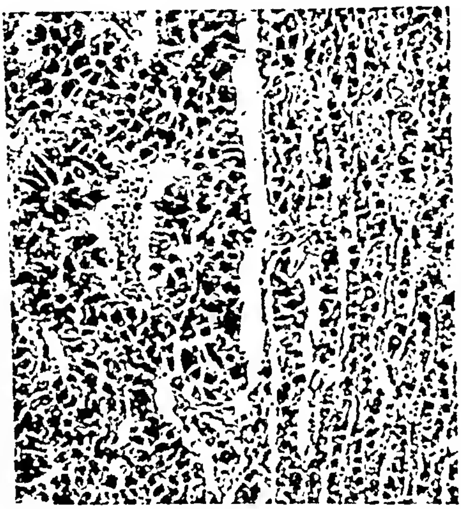


Fig. 4.

Fig. 5.

Fig. 4.—Chromophobe adenoma of the anterior lobe with adjacent parenchyma, showing atrophy by compression.

Fig. 5.—Anterior lobe of the pituitary gland, showing one nodule and fibrous atrophy of the parenchyma.

Lymph Nodes. There are many apparently very recent nodules, together with old lesions. These nodules are small, round, and well-defined accumulations of epithelioid cells with or without lymphocytes, similar to those in tuberculosis. Giant cells are sometimes present. Those with inclusions have darker stained nuclei and cytoplasm than cells withont inclusions. Occasionally inclusions surrounded by a hyaline capsule are seen. In decalcified sections it becomes evident that these calcified inclusions have a particnlar, possibly organic, base which persists after the calcium and iron salts have been dissolved by mineral acids. By fibrosis which starts in the periphery of the nodules in the form of a hyaline shell, the granulation tissue finally passes over into scar tissue.

Liver. The liver shows a moderate degree of cirrhosis with small loose aggregations of epithelioid cells intermingled with a few lymphocytes, sometimes with giant cells, but without the afore-mentioned inclusions. Fat stains reveal a slight, irregular, fatty infiltration.

Spleen. There are microscopically small nodules, variable in appearance, with and without inclusions. There are also calcified inclusions lying free in the pulp. Small ill-defined groups of epithelioid cells apparently represent early stages of the process. The pulp appears slightly fibrotic, and the follicles are rare and atrophic.

Pituitary Gland. Serial sections of the pituitary gland cut in a sagittal plane show a marked excavation of the upper surface. In this region the cell cords of the anterior lobe appear atrophic and run parallel, as is typical of compressed parenchyma of the anterior lobe. However, the pituitary cells in other parts of the anterior lobe also appear atrophic,

being smaller than normal with the chromophilic cells evidently decreased in number. In especially atrophic parts the interstitial fibrous tissue appears thickened and hyalinized. In the middle of the anterior lobe there is a small chromophobe adenoma about 1.5 by 2 mm. in diameter (Fig. 4). The posterior lobe appears particularly small and is almost replaced by tiny nodules with giant cells and calcified inclusions. At the border of both lobes there are a few small colloid-filled follicles. The anterior lobe contains occasional little nodules in the anterior portions of the lobe (Fig. 5). The pituitary stalk is densely infiltrated with small nodules, and only microscopically small parts of the stalk are not destroyed Fig. 6). The capsule of the pituitary gland at the base of the organ is markedly thickened. The infundibulum is likewise infiltrated by tiny nodules, and there are a few on the basal surface of the optic chiasma. The other parts of the floor of the third ventricle were dissected in serial sections, but no essential lesions were found in hematoxylin-eosin stained paraffin section.

Other organs as thyroid, heart muscle, kidneys, and marrow did not show granulomatous lesions in the sections examined.

Fig. 6.—Pituitary stalk infiltrated by granuloma. The black spots represent calcified inclusions.

DISCUSSION

A case of generalized granulomatosis, together with an endocrine disease characterized by genital dystrophia, cessation of the menses, hypotrichosis, and polyuria, has been described. The granulomatosis is characterized by tiny nodules made up of epithelioid cells, lymphocytes, and giant cells differing from those of tuberculosis. These nodules show, in contrast to tubercular ones, that the lymphocytes are frequently intermingled with the epithelioid cells; they do not undergo caseation; they have a particular tendency to get surrounded by a thin hyaline capsule; they have no inclination to coalesce; and they contain peculiar inclusions consisting of an apparently organic base impregnated by calcium and iron salts, while other more rare inclusions seem to be cholesterol crystals. The inclusions give rise to foreign body giant cells, but in addition there are similar looking giant cells which reveal no relation to foreign bodies.

Inclusions, as well as giant cells, may be seen without other components of the granulation tissue, which differs from the findings in any known specific granuloma except the granuloma in sarcoidosis. This granuloma could easily be differentiated from tuberculosis not only by its particular histology as just described but also by negative animal inoculation and cultures.

The involvement of the pituitary and its stalk caused a pituitary disturbance characterized by cessation of menses (substituted, however, by monthly epistaxis), loss of body hair, and polyuria. At autopsy the hypopituitarism manifested itself by atrophy of the ovaries, uterus, tubes, outer genitals, and breasts, and by atrophy of thyroid and adrenals and marked hypotrichosis.

In addition to the destruction of the pituitary stalk and posterior lobe of the pituitary gland by the granuloma, another lesion was found that also might have played a part in the pathogenesis of the hypopituitarism. It was an unusually high and steep dorsum sellae that compressed and stretched the stalk to an abnormal length. How much the chromophobic adenoma in the anterior lobe influenced the picture is hard to say. It might have been compensatory in nature, owing to the atrophy of the anterior lobe, and thus without particular significance as to the development of the pituitary disease.

Since the patient seemed to be normal up to 17 years, with regular menstruation, the anomaly of the dorsum sellae might not have impaired to any great extent the function of the pituitary gland but the damage to the pituitary stalk might have created a "locus minoris resistentiae" where the granuloma easily established itself. On the other hand, the report of diabetes insipidus in other cases of sarcoidosis shows that an involvement of the nervous part of the hypophyseal-diencephalic system is not necessarily based on a previous lesion of this region as described here.

After the granuloma had destroyed the pituitary stalk and the infundibulum, the communication between the pituitary gland and the vegetative centers in the hypothalamus was cut off, the result of which was the atrophy of the anterior lobe. As is known the separation of pituitary gland from the hypothalamus inevitably leads to atrophy of the gland, especially the anterior lobe due apparently to the loss of nerve communications with the vegetative centers in the floor and wall of the third ventricle, and also due to the interruption of channels carrying certain products of the pituitary glands (for instance, gonadotropic hormone) to the diencephalon (Kraus).[3] In view of the long duration of the sarcoidosis it is quite possible that the involvement of the pituitary region by the granuloma reaches far back and that the cessation of the menses might have been the first manifestation of the pituitary disturbance. But it is also possible that the pressure on the pituitary stalk by the anomalous dorsum sellae induced the pituitary disease, which was later much aggravated by the destructive granuloma.

While the disturbance of the water balance certainly resulted from the functional loss of the posterior lobe and pituitary stalk, the genital dystrophia with the hypotrichosis must be referred to the marked atrophy of the anterior lobe.

The atrophy of the thyroid and adrenals also is a result of the pituitary insufficiency as is the generalized atrophy, including the osteoporosis. Since emaciation does not belong to the picture of sarcoidosis, one may suppose that

the generalized atrophy in this case (the patient weighed only 46 kg.) was pituitary in nature.

The vicarious menstruation from the nasal mucosa of many years' duration is hard to explain when one considers the ovaries were entirely atrophic. The later profuse and finally fatal character of the epistaxis might be due to the thrombocytopenia. Since this symptom is not mentioned in other cases of sarcoidosis (while eosinophilia is quite frequent) a causal connection between the sarcoidosis and the blood lesion is not probable, nor is there particular reason to assume pathogenetic relations between the thrombocytopenia and the pituitary disease.

SUMMARY

A case of sarcoidosis (Boeck-Besnier-Schaumann disease) in a woman 41 years of age has been reported. The following microscopic changes have been found characteristic of this granuloma: tiny nodules made up of epithelioid cells, giant cells, and round cells, the latter often intermingled with the epithelioid cells; no tendency to coalesce but rather to be surrounded by a hyaline-fibrous capsule; no tendency to caseation; peculiar inclusions made up of calcium and iron salts compounds deposited in an organic ground substance surrounded by giant cells; occurrence of inclusions, as well as giant cells, alone without other components of the granulation tissue, and finally, conversion into fibrous tissue.

An endocrine syndrome characterized by genital dystrophia, cessation of menses (with vicarious menstruation from the nasal mucosa), hypotrichosis, polyuria, and generalized atrophy has been shown to be caused by the granuloma which has involved the infundibulum, the pituitary stalk, and the pituitary gland itself. An anomaly of the dorsum sellae with pressure on the pituitary stalk probably caused a locus minoris resistentiae for the granuloma. A chromophobe adenoma of the anterior lobe has been interpreted as a compensatory proliferation in an otherwise atrophic organ. A thrombocytopenia causing death could not be referred either to the sarcoidosis or to the endocrine disturbance.

REFERENCES

1. Longcope, W. T.: Sarcoidosis or Besnier-Boeck-Schaumann Disease, J. A. M. A. 117: 1321, 1941.
2. Jüngling, O.: Ostitis tuberculosa multiplex cystica, Fortschr. a.d. Geb. d. Röntgenstrahlen 27: 375, 1919.
3. Kraus, E. J.: Die morphologischen Veränderungen der menschlichen Hypophyse nach Zerstörung der Zwischenhirnbasis bzw. des Hypophysenstiels und deren Folgen, Virchows Arch. f. path Anat. 286: 656, 1932.

TREATMENT OF CONGESTIVE HEART FAILURE IN AMBULATORY PATIENTS WITH AN ORALLY ADMINISTERED MERCURIAL DIURETIC*

JULIUS BURSTEIN, M.D., GEORGE BROWN, M.D., AND CHARLES KLEIN, M.D.
NEW YORK, N. Y.

IN 1940 Batterman, DeGraff, and Rose reported the satisfactory use of an oral preparation of salyrgan-theophylline† in hospitalized patients suffering from congestive heart failure.[1]

It seemed desirable to test the efficacy of this preparation in the treatment of ambulatory patients with congestive heart failure who ordinarily required the frequent administration of intravenous mercurials for maintenance. This study was conducted with patients attending the Morrisania City Hospital Cardiac Clinic.

SUBJECTS, MATERIAL, AND METHODS

Nine patients were selected from a group of 20 who were being treated for congestive heart failure in the out-patient clinic. In all these patients symptoms and signs of failure were not controllable by means of digitalis and the usual oral diuretics (ammonium chloride, aminophylline) alone, but adequate diuresis was obtained by means of intravenous injections of mercurial diuretics.‡ These patients, all ambulatory, had been carried adequately, i.e., nocturnal dyspnea, edema, and weight had been controlled, with one or two weekly injections intravenously of 2 c.c. of a mercurial diuretic, for periods varying from a few months to three years. Of this group of patients, 9 were selected for treatment with the oral preparation of salyrgan-theophylline.† The other 11 patients could not be taken off intravenous mercurial therapy, since the severity of their symptoms did not permit any change of regimen.

Of the 9 patients studied, 6 were men and 3 were women. The age range was from 41 to 69 years: 4 patients suffered from both arteriosclerotic and hypertensive heart disease, 2 from arteriosclerotic heart disease, one from hypertensive heart disease, and 2 from rheumatic heart disease. The urines of all 9 patients, on repeated examinations, showed normal variability in specific gravity, and yielded no pathologic microscopic findings. Blood chemistry determinations were all normal.

For two weeks prior to the change from intravenous to oral mercurial therapy, the 9 patients reported to us, on printed instruction sheets, their urine outputs for the twenty-four-hour period immediately following the mercurial

*From the Cardiac Clinic of the Morrisania City Hospital, Bronx, N. Y.
Received for publication, May 21, 1942.

†Supplied by Department of Medical Research of the Winthrop Chemical Co., labelled S.T.O. 3813. Each tablet contains 80 mg. of salyrgan (30 mg. of mercury and 40 mg. of theophylline).

‡Mercupurin, salyrgan-theophylline. Each cubic centimeter contains the equivalent of 40 mg. of mercury.

injection, and for the twenty-four-hour period just preceding the next injection. This last figure represented the daily output without the influence of a mercurial diuretic, since the measurements were made several days after the effect of the drug had worn off. The patients were instructed to restrict their fluid intakes to 1,000 c.c. daily. They had been receiving 4 to 6 Gm. of ammonium chloride daily as long as they had been on intravenous mercurial therapy.

TABLE I

NAME	AGE	SEX	TYPE OF HEART DISEASE	WEIGHT BEFORE S.T.O.	WEIGHT AFTER S.T.O.	DAILY OUTPUT WITHOUT MERC. DIUR. (C.C.)	DAILY OUTPUT AFTER S.T.O. (C.C.)	DAILY OUTPUT AFTER INTRAV. MERC. (C.C.)	REMARKS
L. C.	60	M	Arteriosclerotic and hypertensive	207	207	1,000	1,500	2,500	Diarrhea
D. S.	49	F	Hypertensive	1-168	166	1,000	3,000	4,500	Onset of diuresis slow
				2-170	171	1,000	Not reported		Severe cramps and diarrhea
D. O.	50	M	Arteriosclerotic	1-175	175	800	1,250	2,000	Mild cramps and diarrhea
				2-181	181	1,000	1,100		No side effects
				3-182	178	1,000	2,000		Severe cramps and diarrhea
L. W.	55	M	Rheumatic and arterio sclerotic	1-161	162	1,000	1,250	5,000	No side effects
				2-153	146	1,000	4,000		No side effects
				3-151	147	750	2,300		No side effects
				4-		Not reported			
				5-		Not reported			
C. T.	41	F	Rheumatic	1-154	152	1,250	3,750	Not reported	Cramps and diarrhea
				2-152	151	1,250	2,750		Cramps and diarrhea
D. S.	67	F	Arteriosclerotic and hypertensive	159	157	750	1,000	2,000	No side effects, but did not feel as comfortable as after intravenous mercurial
S. K.	60	M	Arteriosclerotic and hypertensive	202	199	2,000	3,600	Not reported	No side effects
J. O.	69	M	Arteriosclerotic and hypertensive	142	142	1,500	1,250	Not reported	Cramps and diarrhea
A. N.	64	M	Arteriosclerotic	1-166	164	550	4,000	5,500	No side effects
				2-164	163	550	1,000		No side effects

Then, still keeping the patients on the same fluid intake, and maintaining the administration of ammonium chloride as before, the patients were given 5 tablets of oral salyrgan-theophylline immediately following breakfast on the

day of administration, in place of the intravenous mercurial injection. Again the patients were instructed to measure and report the urine output for the twenty-four-hour period immediately following the oral administration of this preparation, and again for the twenty-four-hour period before the next dose. During this study the patients were weighed just before the tablets were given and again forty-eight hours later.

The results of this study are shown in Table I.

ANALYSIS OF RESULTS

The 9 patients studied received oral salyrgan-theophylline a total of 18 times. The results were not reported three times, leaving a total of 15 reportable trials.

Of the 9 patients studied, one (one trial) showed a decrease in urinary output, about 15 per cent less than when not under the influence of a mercurial diuretic. One patient (two trials) showed no change in output. The other 7 patients (12 trials) all showed an increase in urinary output over that observed when not influenced by the administration of a mercurial diuretic, the increase ranging from 10 to 63 per cent; 7 trials showed an increase of 100 per cent or over; 5 trials, an increase of less than 100 per cent.

All 9 patients reported a greater increase in urinary output after receiving intravenous mercurial than was obtained by the use of oral salyrgan-theophylline. Of 8 measured results after intravenous injection, the range of increase was 66 to 900 per cent, with 7 showing an increase of 100 per cent or more and one an increase of 66 per cent.

Six of the 9 patients developed cramps or diarrhea or both after one to three doses of oral salyrgan-theophylline, which were severe enough to require that the medication be discontinued.

SUMMARY

Nine patients in congestive heart failure, attending an out-patient cardiac clinic, who were known to be well controlled by administration of intravenous mercurial diuretics, were given instead, an oral preparation of mercurial diuretic in a study to determine the usefulness of this medication in ambulatory patients. Seven of the 9 patients (77.7 per cent) showed an increase in urinary output after taking this preparation, as compared with their output when no mercurial diuretic was given. In no case was the increase in urinary output as great as that resulting when the usual dose of a standard mercurial diuretic was given intravenously. Side reactions, chiefly cramps and diarrhea, occurred in 6 of the 9 patients (66.7 per cent) and necessitated discontinuance of administration of the oral mercurial preparation.

REFERENCE

1. Batterman, R. C., DeGraff, A., and Rose, O. A.: Treatment of Congestive Heart Failure With an Orally Administered Mercurial Diuretic, Am. Heart J. 21: 98, 1940.

ADENYLIC ACID AND BACTERIAL GROWTH

SAUL CASPE AND A. G. OSLER*
NEW YORK, N. Y.

DIRECT bacterial infection of the living muscle tissue is not a common phenomenon. Due to its anatomic position the only avenues for muscular infection are through the lymphatics or the blood circulation. Because the latter have their own protective mechanisms, the muscle is thus usually spared from infection. In addition to the above, we believe that the chemical changes and end products occurring during muscular metabolism are inimical to bacterial growth.

In the production of experimental streptococcic arthritis in rabbits, Hadjopoulos[1] and Burbank demonstrated that, whereas the bone marrow, muscular sheaths, and even tendons were infiltrated with streptococci, there was no evidence of the invasion of the muscle tissue. Ammonia and lactic acid are known to be inimical to bacterial growth. However, their concentration in the muscle is not sufficient to inhibit the growths of certain types of bacteria.

The work of Lutwak Mann[2] showed that washed suspensions of *Esch. coli* and of other Bacteriacea dephosphorylated and deaminated adenosine triphosphoric acid, and adenylic acid. These reactions occurred with fresh suspensions as well as with those treated with toluene, and these facts have led Lutwak Mann and others to the conclusion that these reactions are due to bacterial enzymes. What effect the adenine compounds and their dephosphorylated and deaminated derivatives have upon the growth of various types of bacteria was not determined. In order to obtain this information, we devised preliminary experiments.

Our plan, therefore, is to study the effect of adenylic acid upon the growth of various types of bacteria. The usual in vitro experiments do not correspond to the conditions that obtain in normal physiologic processes of the living organism. We desire to approximate these normal body conditions. The work of Freedman and Funk[3] offered application in this connection. These investigators were able to inhibit the growth of bacteria in beef heart broth shaken with charcoal. They postulated the theory that by this procedure a new vitamin was adsorbed upon the charcoal, a vitamin necessary for bacterial growth. By this method, we are afforded an opportunity of observing bacterial growth in a media partially depleted of a bacterial vitamin, and this experimental condition might simulate the conditions occurring in certain parts of the human body.

EXPERIMENTAL

We compare the growth of *B. coli, Staphylococcus aureus, Streptococcus hemolyticus* and *viridans* on (1) starvation media; (2) beef heart infusion; (3) beef heart infusion after treatment with charcoal; (4) beef heart infusion

*Consulting chemist and bacteriologist, New York.

Received for publication, May 1, 1942.

after treatment with charcoal and addition of 0.2 per cent adenylic acid; and (5) starvation media with addition of 0.2 per cent adenylic acid.*

The inoculum for all tests is prepared as follows: Individual organisms are grown in broth culture and are seeded onto agar slants (eighteen hours' growth). The growth is washed with saline, centrifuged, and, again washed four or five times. Then a dilute suspension of the growth is inoculated into a flask containing 10 c.c. of media. The flasks are incubated for ninety-six hours. They are then centrifuged in Hopkins tubes and read. Table I gives the results of this experiment.

TABLE I*

96 HR. GROWTH	GLUCOSE SALT SOLUTION	B.H.I. (DIFCO)	B.H.I. AFTER CHARCOAL	B.H.I. AFTER CHARCOAL PLUS ADENYLIC ACID	G.S.S. PLUS ADENYLIC ACID
B. coli	No growth	B.C. –3.5B H.T. –0.065	B.C. –1.23B H.T. –0.01	No growth	No growth
Streptococcus hemolyticus	No growth	Heavy growth	Very light growth	No growth	No growth
Streptococcus viridans	No growth	Heavy growth	Very light growth	No growth	No growth
Staphylococcus aureus	No growth	H.T. –0.037	H.T. –0.01±	No growth	No growth

*B.C.—Bacteria in billions per cubic centimeter. H.T.—Height of tube. B.H.I.—Beef heart infusion. G.S.S.—Starvation media.

Using a similar procedure the growths of *B. coli* in beef heart infusion, and with 0.2 and 1 per cent adenylic acid are determined. The results are given in Table II.

TABLE II

24 HR. GROWTH	B.H.I. (DIFCO)	B.H.I. PLUS 0.2% ADENYLIC ACID	B.H.I. PLUS 1.0% ADENYLIC ACID	0.5% ADENYLIC ACID (CONTROL)
B. coli	H.T. –0.028	H.T. –0.022	H.T. –0.007+	
				0.001+ (sediment)

The concentration of adenylic acid used in our experiments is probably comparable to the content of adenine compounds of the muscle. The work of at least two workers in this field can be used as reference for such comparison.[4, 5]

It is demonstrated that adenylic acid inhibits the growth of ordinary types of bacteria.

We are indebted to Dr. L. G. Hadjopoulos for his cooperation in these experiments.

REFERENCES

1. Hadjopoulos, L. G., and Burbank, R.: Correlation of Experimental Streptococcic Arthritis in Rabbits With Chronic Rheumatoid Arthritis, J. Bone & Joint Surg. 14: 471, 1932.
2. Lutwak Mann, C.: The Decomposition of Adenine Compounds by Bacteria, Biochem. J. 30: 1405, 1936.
3. Freedman, L., and Funk, C.: Nutritional Factors in the Growth of Yeasts and Bacteria, J. Metabol. Research 1: 457, 1922.
4. Bennet, D. W., and Drury, A. N.: Further Observations Relating to the Physiological Activity of Adenine Compounds, J. Physiol. 72: 288, 1931.
5. Feinschmidt, O.: The Metabolism of Adenosine Triphosphate in Muscles, Biochem. Ztschr. 286: 290, 1936.

474 WEST 238 STREET

*The glucose-salt mixture, which represents our starvation media, is made according to Freedman and Funk. The beef heart infusion is Difco. It is treated with 10 per cent charcoal, and the infusion is heated on a boiling water bath for ten minutes, filtered, and sterilized.

FATAL SALMONELLA INTRACRANIAL INFECTION IN AN INFANT*

Report of a Case

W. E. Bray, M.D., and J. M. Meredith, M.D.
University, Va.

Clinically and bacteriologically the Salmonella group presents many problems.

Clinically, some species or types, as, for example, *S. paratyphi* or *B. paratyphosus A,* infect man only and cause prolonged fever and symptoms indistinguishable from typhoid fever; others, like *S. aertrycke,* cause chiefly gastrointestinal upsets, with sudden onset and short duration, called food poisoning, and are found in both animals and man; and still others, which rarely if ever infect man, like *S. pullorum,* which causes white diarrhea of chickens, are important only economically.[1]

Bacteriologically, there is not only variation in the cultural characteristics, such as in the fermentation of the various sugars, but there is marked variation in the antigen components and consequently in the serologic reactions. Following the work of White[2] (1926-1929) and of Kauffmann[2] (1929-1934) and of later workers[3] in antigen analysis, so many species or types have been described that Jordan and Burrows,[4] in their recent edition of their *Textbook of Bacteriology,* consider that simplification is essential.

The organism cultured from the case here reported was morphologically and culturally like one of the Salmonella group, especially *S. enteritidis,* and was agglutinated to a moderate titer with antisera of *S. aertrycke* and *S. schottmülleri,* but only very slightly with antiserum of *S. enteritidis.* Because of these findings and because of the unusual clinical picture, the culture was sent to the Salmonella Typing Station, located at the University of Kentucky, Lexington, Ky., for identification of the type of the Salmonella. Dr. P. R. Edwards, bacteriologist, Department of Animal Pathology, who has kindly given us permission to use his reports, found the culture to be *S. sandiego,* the antigenic formula of which is IV, V: eh enz$_{15}$.

S. sandiego was first described by Kauffmann,[5] who studied the *S. chester* group and, on the basis of the structure of the H antigen, particularly the β phase, divided the group into two serologic types: *S. chester* and *S. sandiego.*

To the *S. sandiego* type belong:

1. Two original cultures isolated by Professor K. F. Meyer of San Francisco from a case of food poisoning in an outbreak at the San Louis Rey Mission,

*From the Departments of Clinical Pathology and of Neurological Surgery, University of Virginia Hospital and School of Medicine.

Received for publication, May 27, 1942.

where two hundred persons suffered from severe abdominal pain accompanied by vomiting and diarrhea. Most patients recovered within one to two days, but some were ill for ten days or longer.

2. An old laboratory culture, No. 1254, isolated in 1929 in Denmark and mistaken for *S. reading*.

3. Culture CB 42/2, isolated from a healthy pig by Professor E. Hormaeche of Montevideo, Uruguay.

4. Culture b641, obtained from a human being by Professor Hormaeche.

5. Culture 123, obtained by Dr. P. R. Edwards, Lexington, Ky.

The antigenic formulas for these strains are given in Table I, to which we have added the formula for our strain, as determined by Dr. Edwards.

TABLE I

STRAIN	ANTIGEN FORMULA	
Original	IV, XII	eh enz$_{15}$
1254	IV, XII	eh enz$_{15}$
CB 42/2	IV, V, XII	eh enz$_{15}$
641	IV, V, XII	eh enz$_{15}$
123	IV, V, XII	eh enz$_{15}$
UVa	IV, V	eh enz$_{15}$

Dr. Edwards[6] has typed 17 cultures of *S. sandiego:* four from animals, one from sewage, and 12 from man.

The most unusual feature of the following case is the localization of the infection in the subdural space, forming an abscess. The Salmonella infections tend to localize in the bones, joints, and other tissues,[7] and certain members of the Salmonella group, notably *S. enteritidis* or Gärtner's bacillus, have frequently been reported as causing meningitis.[8]

PRELIMINARY NOTE

Baby R. M., Jr., a white male infant, aged 4½ months, University of Virginia History No. 163058, was admitted April 2, 1941, and died April 16, 1941.

The child was born at the University of Virginia Hospital on Nov. 12, 1940, and remained until Dec. 31, 1940. He was a premature child (seven months), forceps being required for delivery. His birth weight was 2,185 Gm. When discharged (Dec. 31, 1940) he was in good condition (weight 2,975 Gm.), although his head was observed to be large, even at that time. At birth the circumference of the head was 35.5 cm.; normally it is 34.7 cm.

The child was next seen on April 1, 1941 (aged 4½ months) in the pediatric out-patient department. He appeared to be very ill. His temperature was 102.2° F. (rectal). The circumference of his head was 53.2 cm. (normal, 40.5 cm.), it being tremendously enlarged. He had vomited for two days previous to his visit to the clinic.

Examination at that time (April 1, 1941) revealed what appeared to be marked hydrocephalus (Fig. 1). The fontanelles were very large and the cranial sutures were widely separated. The child gazed downward constantly in a typically hydrocephalic appearance.

REPORT OF CASE

The following day (April 2) the child was admitted to the hospital on the neurosurgical service for study and possible operation, with a temperature of 103.2° F. (rectal). Each lateral angle of the anterior fontanelle was aspirated and very sanguineous, somewhat purulent, fluid was obtained. This fluid contained 46,900 R.B.C., and 1,980 W.B.C. per cubic millimeter. The following day (April 3) the erythrocytes of the aspirated fluid were diminished in number, but the white blood cells were considerably increased (27,300 R.B.C.; 8,400 W.B.C., 84 per

cent P.M.N.); one week later (April 10) the fluid was very cloudy and contained only 680 R.B.C. but 30,150 W.B.C. per cubic millimeter. Two days before death (April 14) the fluid aspirated through the anterior fontanelle contained 42,600 W.B.C. per cubic millimeter and appeared frankly purulent, with no gross blood discernible.

Examination of the fluid aspirated through the fontanelle on two occasions, for the presence of tumor cells was negative (hematoxylin and eosin stain of centrifuged specimen).

Fig. 1.—Photograph of the patient (twenty-four hours after the first aspiration of fluid through the anterior fontanelle) taken on the day after admission to the hospital (April 3, 1941). Note the very large head, the engorged scalp veins, and the typical downward gaze of the eyeballs so often seen in hydrocephalic infants.

The blood and ventricular fluid Wassermann reactions were negative. Urinalysis revealed many large granular pseudocasts but no red blood cells and no albumin or sugar. An occasional white blood cell per high-power field was seen. Blood counts are shown in Table II.

TABLE II

DATE	HEMOGLOBIN (%)	HEMOGLOBIN (GM. %)	R.B.C. (MILLIONS)	W.B.C.
4/ 3/41 (Day after admission)	62	9.5	3.03	9,300 P.M.N. 28%, Lymph 67%, Mono. 5%
4/ 6/41	65	10.0	5.08	21,000
4/ 8/41			4.16	19,200
4/11/41			3.84	11,200
4/14/41			3.16	34,500

Bacteriologic Examination of Removed Intraventricular or Subdural Fluid.—This was done on several occasions. It soon became evident that none of the ordinary pyogenic bacteria were the cause of the infection. A Salmonella-like

organism was isolated, designated *Salmonella sandiego,* constituting the first instance of an intracranial infection from this organism in our experience.

Course in the Hospital.—The child's condition, as might be expected, was precarious at all times. He died two weeks after admission. His terminal temperature was 101° F (rectal), the temperature readings fluctuating widely, the highest reading being 105.2° F. (rectal) the day after admission. The anterior fontanelle remained very tense at all times except immediately after aspiration. Convulsions occasionally occurred. Sulfathiazole, sulfapyridine, and transfusions of blood were given. Cheyne-Stokes respiration and cyanosis were frequently observed. The immediate cause of death was respiratory failure incident to the overwhelming intracranial infection. A post-mortem examination could not be obtained.

DISCUSSION

An intracranial infection from the organism identified in this case (*S. sandiego*) is very rare. It is possible that the child had a primary (congenital) hydrocephalus on which was engrafted the Salmonella infection, since the child's head was enlarged at birth. Interest also centers on the exact location of the intracranial infection. Was it primarily intraventricular or subdural? The impression of one of us (J. M. M.) who observed the child daily in the ward was that it was primarily subdural, although this can never be proved conclusively since no burr openings were made over the cerebral hemispheres and no post-mortem examination was permitted. It is also quite conceivable that the intracranial involvement began as a purulent meningitis from a primary infection elsewhere in the body (gastrointestinal tract) or from a blood stream infection, proceeding on to gross intraventricular infection (purulent ependymitis) and subdural infection as well.

EPIDEMIOLOGY

Efforts were made to determine the manner in which the infant might have obtained such an unusual infection. Dr. T. S. Englar, Director of the Joint Health Department of the County of Albemarle (Va.) and the City of Charlottesville, kindly instituted a survey of the child's home environment in a nearby country district. His department found that the child had not been breast fed. The mother had been very careful to boil all milk before feeding the baby.

The baby's diet had consisted of boiled milk with Karo syrup added, as well as orange juice and water. He had been fed entirely by bottle and nipple, which were consistently washed and boiled. The milk was usually boiled each morning and night.

The baby's mother continually complained of feeling bad, with headache and nausea, frequently not eating at mealtimes. She did not complain of diarrhea or dysentery at any time so far as is known.

The water supply was from two open springs. Samples of water from these springs were examined in the laboratory of the Department of Preventive Medicine and Bacteriology, University of Virginia, Dr. G. M. Lawson, director. The sample from one spring showed a count of 18 colonies per cubic centimeter, with *B. coli* and *B. aerogenes* confirmed in three tubes of 10 c.c. each; from the

other spring, 38 colonies per cubic centimeter, with *B. aerogenes* confirmed in one tube of 1 c.c., and two tubes of 10 c.c.

The sewage was disposed of through a pit-type toilet located so as not to contaminate the water supply.

The home milk supply was from two cows. One had been sold before the health department survey was made; bacteriologic analysis of milk from the udders of the remaining cow (collected directly into a sterile bottle) showed a count of 56,000 with no lactose fermenters.

SUMMARY

A fatal intracranial infection due to *Salmonella sandiego,* an uncommon type of the genus, is reported in an infant.

A list of the reported cultures of this type, with their antigenic formulas, is given.

This appears to be the first report of this organism since the original description of the type.

Addenda.—Since this paper was prepared, another case of infection with *S. sandiego* in which localization of the infection occurred in the right kidney, and apparently also in the degenerated and necrotic areas of a large uterine fibroid, has been treated in the University of Virginia Hospital. The course of this illness, extending over a period of seventy-six days, was septic in character with blood stream infection, and recovery began apparently only after the infected foci were removed by surgery.

REFERENCES

1. Havens, Leon C.: The Bacteriology of Typhoid, Salmonella and Dysentery Infections and Carrier States, New York, 1935, Commonwealth Fund, page 96.
2. Topley, W. W. C., and Wilson, G. S.: The Principles of Bacteriology and Immunology, ed. 2, Baltimore, 1936, William Wood and Co., page 546.
3. Edwards, P. R.: J. Bact. 32: 259, 1936.
   Edwards, P. R., Bruner, D. W., and Rubin, H. L.: Proc. Soc. Exper. Biol. & Med. 44: 395, 1940.
   Bruner, D. W., and Edwards, P. R.: Am. J. Hyg. 34: 82, 1941.
   Kauffmann, F.: J. Bact. 41: 126, 1941.
4. Jordan, Edwin O., and Burrows, William: Text Book of Bacteriology, Philadelphia, 1942, W. B. Saunders Co., page 350.
5. Kauffmann, F.: Acta path. et microbiol. Scandinav. 17: 429, 1940.
6. Personal communication.
7. Goulder, N. E., Kingsland, Margaret F., and Janeway, C. A.: New England J. Med. 226: 127, 1942.
8. Meyer-Rohn, J.: Deutsche med. Wchnschr. 66: 1193, 1940. (Gärtner bacillus.)
   Wengeler, J.: Med. Klin. 36: 1217, 1940. (Gärtner bacillus.)
   Guthrie, K. J., and Anderson, T.: Brit. M. J. 1: 193, 1941. (Gärtner bacillus and meningococcus.)
   Wilsdon, K. F., and Gibson, A.: Lancet 1: 665, 1941. (*S. london.*)

EFFECT OF INJECTION OF TISSUE EXTRACTS ON THE NUMBER OF BLOOD PLATELETS*

Alfred Uihlein, M.D., Rochester, Minn.

THIS investigation was stimulated by experiments[3, 19-21] which indicated that a thrombocytolytic agent, "thrombocytopen," can be extracted from spleens of patients who have idiopathic hemorrhagic purpura and was undertaken at the suggestion of Dr. H. Z. Giffin and Dr. J. deJ. Pemberton. Some reports in the literature demonstrated that a substance can be isolated which not only decreases the number of circulating blood platelets of animals,[2, 18, 19] but even alters the normal configuration of the megakaryocytes when cultured in vitro.[14-17] The thrombocytolytic substance apparently reduces the number of platelets, but experimentally, it does not produce purpura. During the progress of this study, several investigators[1, 4, 10, 13] reported their inability to isolate thrombocytopen, while others confirmed its presence.[5, 7, 12]

METHOD

For the purpose of investigation, extracts of 28 spleens, together with normal hepatic and lymphoid tissue from one patient, were prepared. Thirteen spleens were from patients who had idiopathic hemorrhagic purpura, five from patients who had hemolytic icterus, five from those who had Banti's disease, and one from a patient who had Hodgkin's disease. There were four normal spleens. All but three of the spleens were obtained from the operating room, and each was prepared for extraction within thirty minutes after splenectomy. Three spleens and the hepatic and lymphoid tissues were obtained within an hour and a half after death.

The tissues were extracted according to the procedure outlined by Troland and Lee.[21] Briefly, each tissue was ground in a medium-fine meat grinder and weighed. It was then placed in a jar containing acetone, the volume of which was twice the weight of the tissue. The container was sealed and permitted to remain at room temperature for about four weeks, gently agitated at least once a day. Three spleens were extracted in commercial acetone, while all other tissues were placed in reagent acetone. After extraction the mixture was filtered and centrifuged. The acetone then was removed by distillation. The residue was brown and gummy. It was mixed with a sufficient amount of distilled water to make 1 c.c. represent 1 Gm. of tissue. This aqueous mixture again was filtered and the filtrate was placed in the refrigerator where it remained for about two

*From the Mayo Clinic, Rochester.

Abridgment of thesis submitted to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of *Master of Science* in Surgery.

Work done in the Institute of Experimental Medicine, the Mayo Foundation, Rochester.

Received for publication, May 25, 1942.

weeks. If a precipitate formed, gentle agitation and passage through a Zeiss filter cleared the filtrate. A preservative was not added.*

At first, experiments were conducted on rabbits that averaged 2 kg. After a few experiments, however, rabbits were rejected because it was difficult to obtain control platelet counts from them, and rats weighing 200 Gm. (Wistar strain) were used. Control platelet counts were carried out for from one to three days prior to injection of the extract. Usually 1 c.c. of the extracted material, the equivalent of 1 Gm. of tissue, was injected intravenously; however, as much as 20 c.c. were given. Subcutaneous and intraperitoneal injections also were tried in a few instances. Following intravenous injection the number of platelets was determined at hourly intervals for the first six hours, and thereafter, every six hours until no further variation in the number of platelets was noted. Each animal received only one injection.

The method used for counting the platelets differed slightly from that of Troland and Lee.[21] A 2 per cent solution of sodium citrate in normal saline solution, as described by Ottenberg and Rosenthal but modified by the Division of Clinical Pathology of the Mayo Clinic, was used instead of the Rees-Ecker solution, as the latter diluent contained too many platelet-like artifacts. The solution of sodium citrate contained 2.26 Gm. of sodium citrate, 1.8 Gm. of sodium chloride, and 220 c.c. of distilled water. The solution was thrice filtered into a sterile flask. The diluent was always freshly prepared and was used at room temperature.

Blood was obtained by cutaneous puncture of the ear of the animal. The first few drops were discarded,[8, 9] and specimens were not collected unless the blood flowed freely without compression of the ear. However, gentle compression of the ear was at times necessary after injection of the extract because the blood flow was decreased; the cause of this was not apparent. The blood was drawn up to the 0.5 mark in an erythrocyte pipette and diluted to 1.1 with the solution of sodium citrate. This mixture was agitated gently by hand for three minutes. Following a five-minute quiescent period a third of the contents in the bulb of the pipette was expelled, and succeeding drops were carefully placed under each half of the cover slip on a modified Neubauer counting chamber. The chamber was then placed on a moistened piece of filter paper in a Petri dish for ten minutes to avoid desiccation and to permit settling of the cells. The number of platelets and erythrocytes in eighty small squares was counted with a high power objective and a 10× ocular. Computation was made in the usual manner. Only a variation of ±200,000 in each count was considered significant.

RESULTS

Extracts of 28 human spleens, and one extract of hepatic and one of lymphoid tissue were prepared. Of these, four spleens, the liver and lymph nodes were obtained as controls. Various responses were obtained. The first two spleens extracted were from patients who had idiopathic hemorrhagic purpura; these spleens were only slightly larger than normal (Table I). The extracts of these two spleens, when injected, produced a dramatic fall of number of thrombocytes of the first rat from a preinjection level of 700,000 in each cubic

*I wish to express my appreciation to Dr. A. E. Osterberg for his assistance in preparation of the tissue extracts.

millimeter of blood to 40,000 in twenty-four hours (Fig. 1). Blood smears, stained with Wright's stain, confirmed the presence of thrombopenia. The thrombopenia was prolonged for seventy-two hours, after which the number of platelets gradually returned to the preinjection level. Moreover, injection of these extracts was followed by a similar response in eleven other normal rats, each of which weighed 200 Gm. All the animals appeared ill at the height of the thrombopenia. At the end of approximately a week these extracts became extremely toxic. Subsequently all animals died within six hours after the injection.[11] Hemorrhage or purpuric spots did not appear, nor could any definite change be noted in the bleeding or coagulation time. The number of erythrocytes did not vary significantly.

TABLE I

CASES IN WHICH THE SPLEEN WAS EXTRACTED

CASE	SEX	CONDITION	WEIGHT OF TISSUE EXTRACTED (GM.)
1	F	Hemorrhagic purpura, idiopathic	115
2	M	Hemorrhagic purpura, idiopathic	110
3	F	Hemorrhagic purpura, idiopathic	80
4	F	Hemorrhagic purpura, idiopathic	90
5	F	Hemorrhagic purpura, idiopathic	55
6	F	Hemorrhagic purpura, idiopathic	64
7	F	Hemorrhagic purpura, idiopathic	120
8	F	Hemorrhagic purpura, idiopathic	100
9	M	Hemorrhagic purpura, idiopathic	117
10	F	Hemorrhagic purpura, idiopathic	104
11	M	Hemorrhagic purpura, idiopathic	95
12	F	Hemorrhagic purpura, idiopathic	78
13	F	Hemorrhagic purpura, idiopathic	195
14	F	Hemolytic icterus	363
15	F	Hemolytic icterus	188
16	F	Hemolytic icterus	160
17	M	Hemolytic icterus	460
18	M	Hemolytic icterus	180
19	M	Banti's splenomegaly	350
20	M	Banti's splenomegaly	320
21	M	Banti's splenomegaly	300
22	M	Banti's splenomegaly	382
23	M	Banti's splenomegaly	282
24	M	Hodgkin's splenomegaly (grade 4 sarcoma type)	590
25	F	Normal spleen	36
26		Normal spleen	140
27		Normal spleen	125
28		Normal spleen	125
29		Normal liver	490
		Normal lymph nodes	25

Three other extracts of spleens obtained from patients who had hemorrhagic purpura produced only a small transitory decrease in the circulating platelets in six hours; one other extract of a spleen from a patient who had hemolytic icterus produced a definite decrease in the number of platelets, which lasted only a few hours. None of these produced the striking thrombopenia of the first two extracts (Fig. 1). These extracts, however, never became toxic, although several animals received injections. The extracts from the remaining tissue did not produce any significant decrease of the number of blood platelets or erythrocytes after injection (Fig. 1). Even injection of larger amounts of the extracts, as high as 20 c.c. failed to reduce the number of platelets. However, all animals ap-

peared apathetic for short periods after the injections, a result suggestive of an indeterminate type of reaction.

COMMENT

Although thrombocytopen could not be extracted consistently from all the spleens of patients who had hemorrhagic purpura, its presence was indicated in some of them. Five extracts of the 13 spleens from patients who had idiopathic hemorrhagic purpura produced a certain amount of thrombolytic activity. Moreover, one of five spleens from patients who had hemolytic icterus apparently contained a thrombocytolytic substance (Table II). The fact that all animals

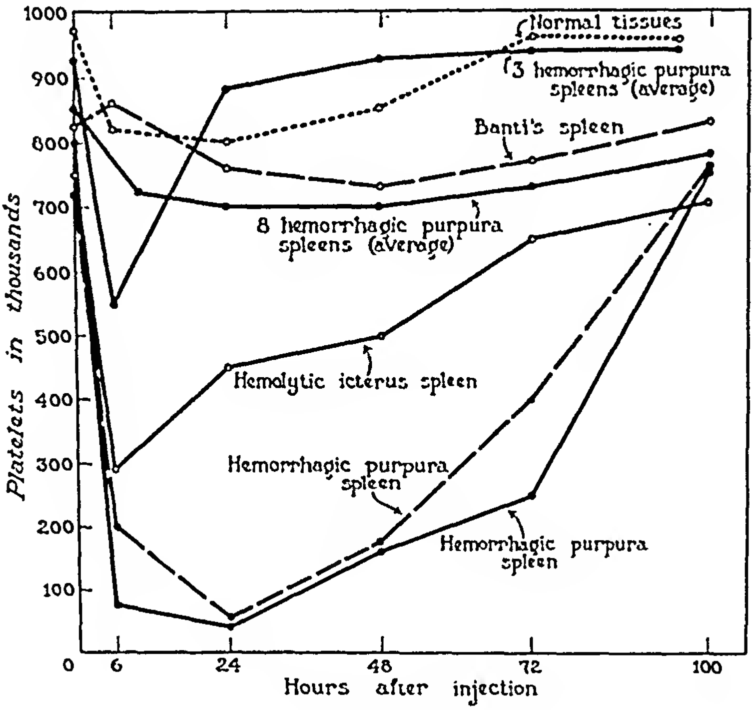


Fig. 1.—Effect of injections of various tissue extracts on the circulating blood platelets of the albino rat.

were apathetic after the injection of the extracts suggests an anaphylactoid response, probably nonprotein in nature since the acetone is believed to remove all the protein. The extracts were impure and the lipoid fraction may have contained substances which produced this effect.

Neither extraction with reagent acetone or with commercial acetone nor distillation of the acetone fraction by various methods seemed to influence the thrombolytic activity of the extracts.

No explanation can be given for the toxic manifestations encountered with the first two extracts used. Dilution of the material and filtration through a Zeiss filter failed to diminish the toxicity. It is not likely that failure of the number of blood platelets to decrease could be attributed to the citrate solution. Platelets counted after dilution with a 2 per cent solution of sodium citrate remained consistently constant prior to injection, and this result suggested that the method was satisfactory. With the Rees-Ecker stain a platelet count that is too high rather than too low is likely to be obtained.

Rats were used instead of rabbits principally because the control platelet counts were more constant and because these animals were more available. Since most of the data in the literature on this problem were derived from experiments on rabbits, some investigators may criticize these results as incomparable with the results of earlier studies. It is doubtful, however, whether the type of laboratory animal used is a factor in influencing the results, since rabbits also were given injections of the extracts in the first few experiments. With rabbits the response of the platelets was not unlike that found among rats.

TABLE II

RESPONSE OF PLATELETS TO INJECTION OF TISSUE EXTRACT

CASES IN WHICH SPLEEN WAS EXTRACTED		DECREASE IN NUMBER OF PLATELETS	
CONDITION	NUMBER	100,000 AND MORE	LESS THAN 100,000
Idiopathic hemorrhagic purpura	13	5	8
Hemolytic icterus	5	1	4
Banti's disease	5		5
Normal spleen	4		4
Normal liver	1		1

SUMMARY

The results of this study neither clearly demonstrate nor definitely deny the existence of a thrombocytolytic substance, "thrombocytopen," in extracts of spleens from patients who had idiopathic hemorrhagic purpura. Because of the inconsistency of the results, the study indicates that a better method of extraction is essential. Twenty-eight spleens, liver tissue, and lymphoid tissue were obtained from patients and the thrombocytolytic substance was extracted.

Thirteen spleens from patients who had hemorrhagic purpura were extracted. Extracts from two produced a considerable drop in the circulating blood platelets; in three they produced a moderate decrease, while the rest gave no response. Five spleens from patients who had hemolytic icterus were extracted; the extract of one spleen apparently lowered the number of platelets. This response was repeated in other animals and was not eliminated by heating the extract.

The decrease of the number of platelets and the lethargic state of the animals after injection suggested the presence of anaphylaxis or a histamine-like reaction, since the symptoms appeared shortly after administration of the extract and disappeared within a few hours.

REFERENCES

1. Colmer, M. L., and Mersheimer, W. L.: Relation of Splenic Extract to Etiology of Essential Thrombopenia, Arch. Surg. 43: 422, 1941.
2. Hittmair, Anton: Das Blutplättchen im Spiegel des Schrifttums der letzten 10 Jahre, Folia haemat. 59: 50, 1938.
3. Hobson, F. C. G., and Witts, L. J.: Platelet Reducing Extracts of the Spleen, Brit. M. J. 1: 50, 1940.
4. Major, R. H., and Weber, C. J.: Is There a Platelet-Reducing Substance in the Spleen of Thrombocytopenic Purpura? J. LAB. & CLIN. MED. 25: 10, 1939.
5. Otenasek, Frank, and Lee, F. C.: Further Observations on Thrombocytopen, J. LAB. & CLIN. MED. 26: 1266, 1941.
6. Ottenberg, Reuben, and Rosenthal, Nathan: A New and Simple Method for Counting Blood Platelets, J. A. M. A. 69: 999, 1917.
7. Paul, J. T.: The Effect of Splenic Extracts From Cases of Essential Thrombocytopenic Purpura on Platelets and Hematopoietic Organs of Rabbits (Abstr.), Proc. Central Soc. Clin. Research 14: 55, 1941.

8. Peirce, E. G., and Pemberton, Ralph: The Red Cell Count in Arthritis, Arch. Int. Med. 39: 421, 1927.
9. Pemberton, Ralph: The Etiology and Pathology of Arthritis, J. A. M. A. 87: 1253, 1926.
10. Pohle, F. J., and Meyer, O. O.: Inability to Demonstrate a Platelet Reducing Substance in an Acetone Extract of the Spleen From Patients With Idiopathic Thrombocytopenic Purpura, J. Clin. Investigation 18: 537, 1939.
11. Rees, H. M., and Ecker, E. E.: An Improved Method for Counting Blood Platelets, J. A. M. A. 80: 621, 1923.
12. Rose, Hugh, Jr., and Boyer, L. B.: Thrombocytopen: A Confirmatory Report, J. Clin. Investigation 20: 81, 1941.
13. Tocantins, L. M.: No Platelet-Destroying Action in Extracts of the Spleen and Urine of Patients With Chronic Thrombopenic Purpura, Proc. Soc. Exper. Biol. & Med. 42: 485, 1939.
14. Torrioli, M., and Belleli, D.: Studi sulla biologia dei megacariociti sopravviventi in vitro; azione di estratti di milza a concentrazioni scalari, Il Policlinico (sez. med.) 42: 214, 1935.
15. Torrioli, M., and Galeazzi, M.: Studi sulla biologia dei megacariociti sopravviventi in vitro: azione di estratti di muscolo e di fegato, Il Policlinico (sez. med.) 41: 647, 1934.
16. Torrioli, M., and Puddu, V.: Studi sulla biologia dei megacariociti sopravviventi in vitro; azione del sangue venose splenico, Il Policlinico (sez. med.) 42: 129, 1935.
17. Torrioli, M., and Puddu, V.: Studi sulla biologia dei megacariociti sopravviventi in vitro, Il Policlinico (sez. med.) 41: 245, 1934.
18. Torrioli, M., and Pusic, G.: Azione piastrinopenica elettiva di dosi massive di estratto splenico, Boll. Soc. ital. biol. sper. 9: 662, 1934.
19. Torrioli, Mario, and Puddu, Vittorio: Recent Studies on Pathogenesis of Werlhof's Disease, J. A. M. A. 111: 1455, 1938.
20. Troland, C. E., and Lee, F. C.: Thrombocytopen: A Substance in the Extract From the Spleen of Patients With Idiopathic Thrombocytopenic Purpura That Reduces the Number of Blood Platelets, J. A. M. A. 111: 221, 1938.
21. Troland, C. E., and Lee, F. C.: A Preliminary Report on a Platelet-Reducing Substance in the Spleen of Thrombocytopenic Purpura, Bull. Johns Hopkins Hosp. 62: 85, 1938.

A PRELIMINARY REPORT ON THE TREATMENT OF BACILLARY DYSENTERY WITH SUCCINYL SULFATHIAZOLE*

EDGAR J. POTH, PH.D., M.D., BEACH M. CHENOWETH, JR., M.D., AND F. LOUIS KNOTTS, M.D., BALTIMORE, MD.

THE introduction of sulfonamide therapy has been the most significant recent advance in the treatment of bacillary dysentery. The dysentery organisms are particularly susceptible to the antibacterial action of several members of this series of drugs. Sulfapyridine[1, 2] and sulfathiazole[3-7] are probably the most effective sulfonamides reported so far. They would be entirely satisfactory if they were less toxic.

Cooper and Keller[8] showed that a single dose of 2 mg. of sulfathiazole given orally at the time of intraperitoneal injection of a suspension of 1,000 minimal lethal doses of *Shigella paradysenteria* Flexner protected mice against the infection. Under similar conditions sulfanilamide was ineffectual.

*From the Department of Surgery and the Department of Pediatrics, the Johns Hopkins University School of Medicine, Baltimore.
This work was supported by a grant from Sharp & Dohme, Philadelphia, Pa.
Received for publication, June 6, 1942.

The successful use of various sulfonamides would indicate that many of these compounds are effective against bacillary dysentery when they are absorbed slowly enough from the gastrointestinal tract to allow the concentration of the drug to reach bacteriostatic levels in the bowel. An important property of an active compound ideally suited to the treatment of bacillary dysentery requires, therefore, that the drug be sparingly absorbed from the bowel. This property will keep the drug where it can be directly active against the bacteria and will minimize general toxicity.

TOXICITY OF SULFAPYRIDINE, SULFATHIAZOLE, AND SULFANILYLGUANIDINE

A review of the literature on the use of sulfapyridine, sulfathiazole, and sulfanilylguanidine shows that these compounds cause toxic reactions when used to treat bacillary dysentery.

Sulfapyridine is the most toxic. Ravenel and Smith[2] state, "In a few patients sulfapyridine produced marked restlessness; this was controlled with phenobarbital."

Welch[9] found more severe manifestatiosn of toxicity: "Sulfapyridine administration is followed by symptoms of nausea and vomiting which occur to varying degrees in 30 to 40 per cent of children under treatment. Too frequently the vomiting makes the therapy disagreeable and too often is so severe that it necessitates discontinuing the drug."

The toxic manifestations due to sulfathiazole are less severe. One patient we treated developed a rash which necessitated discontinuance of sulfathiazole. A more serious sequence was reported by Taylor.[4] An infant, 4 months of age, received 0.9 Gm. of sulfathiazole in the presence of a slight jaundice. The jaundice increased and the drug was discontinued. The child died twenty-three days later of the bacillary dysentery. Although the jaundice might have been due to multiple transfusions of blood, it was for fear of a drug reaction that sulfathiazole was withheld.

Of the three drugs used most widely in the treatment of bacillary dysentery, sulfanilylguanidine appears least toxic. Lyon[10] observed mild toxic manifestations, consisting of (1) disturbances of sensorium, (2) conjunctivitis, and (3) "discoloration" of the skin. These evidences of mild toxicity occurred in four of the 23 cases treated.

SUCCINYL SULFATHIAZOLE AS A CHEMOTHERAPEUTIC AGENT LOCALLY ACTIVE IN THE BOWEL

The experimental and clinical foundation for the use of succinyl sulfathiazole in the treatment of bacillary dysentery was laid by Poth, Knotts, Lee, and Inui[11-13] These authors demonstrated that this compound has much greater local activity against the coliform organisms in the bowel than sufathiazole and sulfanilylguanidine. The physical character of feces is profoundly altered by the action of succinyl sulfathiazole. The feces become practically odorless.

Furthermore, it has been shown that the toxicity of this drug is unusually low. Approximately 5 per cent of the ingested compound is excreted by the kidneys when diarrhea is absent. In the presence of diarrhea an even smaller proportion of the therapeutic dose is recovered from the urine.

TABLE I

RESULTS OF TREATING BACILLARY DYSENTERY IN CHILDREN WITH SUCCINYL SULFATHIAZOLE

NAME	WT. IN KG.	AGE	DURATION DIARRHEA BEFORE TREATMENT	ORGANISM	MAXIMUM TEMPERATURE	NO. OF HOURS TREATMENT REQUIRED TO LOWER TEMPERATURE TO 37° C.	NO. STOOLS DAILY BEFORE TREATMENT	NO. STOOLS ON DAYS OF TREATMENT					DOSAGE GM./KG./DAY*	NO. DAYS TREATED
								1	2	3	4	5		
MN	15.0	3 yr.	8 hr.	Sonne	41° C.	12	Frequent	7	1	0	0	1	0.20 (a)	2½
CL	11.0	2 yr.	12 hr.	Flexner	40° C.	24	Frequent	5	2	1	0	1	0.25 (a)	2
KS	22.4	8 yr.	12 hr.	Flexner	40° C.	18	8	6	0	0	1	0	0.20	5
DT	10.4	3 yr.	24 hr.	Flexner	39.8° C.	12	10-12	6	2	1	3	0	0.3 (b)	4
TY	12.4	3 yr.	24 hr.	Flexner	40° C.	36	8	1	0	2	3	2	0.25 (b)	8
GY	13.0	2 yr.	24 hr.	Sonne	40° C.	12	Many	2	0	1	0	2	0.25	5
JE	11.5	2 yr.	5 days	Flexner	38.5° C.	12	10-15	4	0	3	2	0	0.25 (a)	5
BL	8.2	10 mo.	9 days	Flexner	39.6° C.	24	Many	5	3	1	4	4	0.5 (c)	1½
RR	8.9	14 mo.	13 days	Sonne	38.8° C.	16	8-10	3	0	1	2	1	0.5 (c)	3
MY	3.0	8 wk.	14 days	Flexner	40° C.	24	12	4	6	2	1	3	1.0	6

*All daily dosages are divided into six equal portions and administered at four-hour intervals.
(a)—1 Gm. as an initial dose.
(b)—1.5 Gm. as an initial dose.
(c)—0.75 Gm. as an initial dose.

TREATMENT OF BACILLARY DYSENTERY WITH SUCCINYL SULFATHIAZLE*

The usual general symptomatic treatment was given all patients. This regimen included the administration of adequate parenteral fluids and electrolytes to combat dehydration when the need for this was indicated. No patients received transfusions of whole blood or plasma for the treatment of the dysentery. One patient did receive a single transfusion of whole blood because of a long-standing nutritional anemia. The diagnosis of bacillary dysentery was confirmed bacteriologically in all cases included in this study.

The drug was given by mouth, and the dosage varied from 0.25 Gm. to 1.0 Gm. per kilogram daily, divided into six equal portions. The length of treatment varied from two to seventeen days. As indicated in Tables I and II, there were no failures and no deaths. The patients ranged in age from 8 weeks to 83 years.

TABLE II

BACILLARY DYSENTERY IN ADULTS TREATED WITH SUCCINYL SULFATHIAZOLE

NAME	AGE (YR.)	DURATION OF DIARRHEA BEFORE TREATMENT STARTED	ORGANISM	NO. STOOLS BEFORE TREATMENT	STOOLS DAILY WITH TREATMENT					DOSAGE GM./KG./DAY	NO. DAYS TREATED
					1	2	3	4	5		
ST	21	3 days	Shiga	9	3	2	3	1	1	0.25 (a)	4
RS	22	4 days	Sonne	12	4	3	2	2	2	0.25	4
RS	19	4 days	Flexner	15	9	5	3	1	1	b	4
WW	29	7 days	Flexner	15-18	14	9	6	2	1	b	4
MM	23	2 days	Flexner	20-25	14	9	3	1	1	b	4
HS	23	6 days	Flexner	20-25	14	4	2	1	1	b	4
JO	25	1 day	Not typed	12-15	12	3	1	1	1	b	4
SS	30	2 days	Flexner	25-30	20	7	8	2	1	b	6
DN*	83	32 days	Flexner	10-15	4	1	8	8	4	b	17
HY	57	3 mo.	Flexner	Many	4	1	2	1	1	0.25 (a)	14

*This patient was in extremely poor condition, with malnutrition and decubitus ulcers, and showed a rash following sulfathiazole therapy without benefit to dysentery before succinyl sulfathiazole treatment was instituted. Treatment was continued much longer than necessary.

(a)—0.25 Gm. per kilogram as a single initial dose.

b —0.5 Gm. per kilogram daily for two days, then 0.25 Gm. per kilogram daily. Daily dosage is divided into six equal portions and given at four-hour intervals.

The data recorded in Table I indicate that the response of children and infants suffering from bacillary dysentery when treated with succinyl sulfathiazole is prompt whether treatment is begun early or late in the disease. The response is equally good with the smaller doses as with the larger. The temperature returned to normal in twenty-four hours or less in all instances except one. In this case the temperature became normal in thirty-six hours. The results of treating ten adults with bacillary dysentery with succinyl sulfathiazole are summarized in Table II. The response to therapy is again prompt regardless of the duration of the disease.

The number of patients in this series was insufficient to determine accurately the minimum dosage of drug and the shortest period of therapy required for the successful treatment of bacillary dysentery with succinyl sulfathiazole. It is obvious, however, that in most instances unnecessarily large quantities of drug were given for longer periods than required. In fact, the response was better in those patients receiving the smaller doses. Bacteriologic studies showed the *Shigella paradysenteriae* organisms to be especially susceptible to the anti-

*The succinyl sulfathiazole (sulfasuxidine) used in this study was supplied by Sharp & Dohme, Philadelphia, Pa.

bacterial action of succinyl sulfathiazole. The dysentery organisms ordinarily disappeared from stools within forty-eight hours, whereas significant lowering of the coliform bacteria required two or three days of additional therapy.

It is especially significant that the response to succinyl sulfathiazole is immediate, although the disease has been present for as long as three months before treatment is undertaken. This result is in contradistinction to the observations made by Lyon and Marshall regarding the efficacy of sulfanilylguanidine. As stated by Lyon:[14] "The drug appears to be most efficacious when administered during the first 3 to 4 days after onset of fever or diarrhea. Although the percentage of failures may be higher when the drug is not given until after the fifth day of illness, it has been observed to be effective at times even when given later in the disease."

Marshall, Bratton, Edwards, and Walker[15] say: "Ten children were treated late in the disease, the institution of chemotherapy varying from the 4th to the 14th day of the disease. In this group the results were not uniform. Some cases showed the same striking improvement that occurred in the children treated early, but the majority ran a course uninfluenced by the administration of sulfanilylguanidine."

It appears that sulfanilylguanidine is most effective against bacillary dysentery if the treatment is started early. It may fail to influence the course of the disease if treatment is delayed.

DISCUSSION

While the results of treating bacillary dysentery with the sulfonamides are so good in the United States, it must be realized that this outcome is partly due to the excellent state of nutrition usually encountered here. In areas where states of poor nutrition exist, especially among children, mere bacteriostasis does not give such brilliant results, because the diarrheas of the specific bacillary dysenteries are complicated by malnutrition. Under these circumstances attention to general care, fluid, and electrolyte replacement, and nutrition must be held of equal importance with specific drug therapy.

Under ordinary conditions of our national civilization, sanitary safeguards and food protection are developed to a high degree and bacillary dysentery among adults is not a serious problem. In the armed forces, however, men in strange climates and frequently in unsanitary surroundings are unduly exposed, and infection of the adult becomes a problem of major importance. Because of the apparent great efficacy of succinyl sulfathiazole in bacillary dysentery and the absence of toxic symptoms due to the administration of this drug, it would be highly desirable to consider the use of succinyl sulfathiazole as a prophylactic agent. A small quantity of drug given in the daily ration of men in areas of heavy exposure might offer protection. Such a protective dose might be surprisingly small; and in the absence of any effect on the sensorium, as determined by reaction time, and so on, the drug could be administered to great advantage.

The doses given in the cases of bacillary dysentery reported here may well be much larger than is required. No toxicity to the drug has been observed in any of these patients, and there were no instances where the drug failed to ar-

rest the disease in a very short time. Since the minimum effective therapeutic dose of succinyl sulfathiazole has obviously not been established, a satisfactory prophylactic dose might be surprisingly small.

Because sulfathiazole is highly effective in bacillary dysentery, because the activity of succinyl sulfathiazole is probably due to the degradation product, nascent sulfathiazole, and because succinyl sulfathiazole can be maintained in high concentration in the bowel with only slight absorption and without the development of toxic manifestations, it is not surprising that succinyl sulfathiazole in tolerated therapeutic doses should have a pronounced antibacterial activity against the organisms of bacillary dysentery. In all fairness it should be stated that during the past summer bacillary dysentery was not prevalent, and the disease might have been relatively mild. This communication should, therefore, be regarded as a preliminary report. Kirby and Rantz[16] have, however, succeeded in curing bacillary dysentery carriers with succinyl sulfathiazole.

SUMMARY

The treatment of bacillary dysentery with a new chemotherapeutic agent, succinyl sulfathiazole, is presented. The drug is equally effective in both the acute and the more chronic forms of the disease. All cases of bacillary dysentery treated responded promptly. The administration of the drug caused no untoward toxic manifestations.

We wish to thank Dr. Samuel Revell, of the University of Maryland School of Medicine, and Lt. David W. Exley, of Camp Jackson, South Carolina, for the privilege of including in this report cases of bacillary dysentery treated under their direction.

REFERENCES

1. Reutler, R., and Marberg, K.: Note on the Treatment of Acute Bacillary Dysentery With Sulfapyridine, Brit. M. J. p. 277, Feb. 22, 1941.
2. Bell, G. J.: Treatment of Bacillary Dysentery With M & B 693, Lancet 241: 101, 1941.
3. Anderson, E. V.: The Chemotherapy of Infectious Diarrhea With Sulfathiazole, J. Pediat. 18: 732, 1941.
4. Taylor, G.: Sulfathiazole Therapy of Infantile Diarrhea, J. Pediat. 18: 469, 1941.
5. Lawrence, C. A.: Bacteriostatic Effects of Sulfanilamide, Pyridine, Thiazole Derivatives Upon Colon-Typhoid-Dysentery Group, Proc. Soc. Exper. Biol. & Med. 44: 162, 1940.
6. Ravenel, S. F., and Smith, D. L., Sr.: The Chemotherapy of Bacillary Dysentery, South. M. J. 34: 509, 1941.
7. Cooper, M. L., Zucker, R. L., and Wagoner, S.: The Treatment of Infantile Dysentery With Sulfathiazole, J. A. M. A. 117: 1520, 1941.
8. Cooper, M. L., and Keller, H. M.: Sulfonamide Drugs in the Treatment of Experimental Dysentery Infection in Mice, J. Pediat. 18: 460, 1941.
9. Welch, S. H.: In Discussion, South. M. J. 34: 510, 1941.
10. Lyon, G. M.: Sulfanilylguanidine: Treatment of Acute Bacillary Dysentery, U. S. Nav. M. Bull. 39: 286, 1941.
11. Poth, E. J., and Knotts, F. L.: Succinyl Sulfathiazole. A New Bacteriostatic Agent Locally Active in the Gastrointestinal Tract, Proc. Soc. Exper. Biol. & Med. 48: 129, 1941.
12. Poth, E. J., Knotts, F. L., Lee, J. T., and Inui, F.: Bacteriostatic Properties of Sulfanilamide and Some of Its Derivatives. I. Succinylsulfathiazole, a New Chemotherapeutic Agent Locally Active in the Gastrointestinal Tract, Arch. Surg. 44: 187, 1942.
13. Poth, E. J., and Knotts, F. L.: Clinical Use of Succinyl-sulfathiazole, Arch. Surg. 44: 208, 1942.
14. Loc. cit., page 293.
15. Marshall, E. K., Jr., Bratton, A. C., Edwards, L. B., and Walker, Ethel: Sulfanilylguanidine in the Treatment of Acute Bacillary Dysentery in Children, Bull. Johns Hopkins Hosp. 68: 101, 1941.
16. Kirby, W. M. M., and Rantz, L. A.: The Treatment of Typhoid and Dysentery Carriers With Succinyl Sulfathiazole, J. A. M. A. 119: 615, 1942.

A NEW HYPOTHESIS OF THE PRODUCTION OF THE T WAVE IN THE ELECTROCARDIOGRAM BASED ON ELECTROKINETIC PHENOMENA*

J. Roscoe Miller, M.S., M.D., F.A.C.P., and Roy F. Dent, B.S., M.D.
Chicago, Ill.

THE purpose of this paper is to describe a possible mechanism for the production of the T-wave component of the electrocardiogram and the experimental procedure followed in its preliminary study. This study was begun with the hope that a more logical and concrete explanation of the electrobiologic phenomena responsible for this phase of the cardiogram could be found than those explanations now current.

In general, it is accepted that the T wave represents the disappearance of the electric disturbance responsible for the QRS deflection, which phenomenon has been variously termed repolarization, return to the resting state, end deflection of the QRS, etc. The exact manner in which this occurs, however, is disputed. The earlier hypothesis states that the character of the T wave is determined by the vectorial summation of the last portions of two out-of-phase monophasic waves.[1-5] More recently, with the better understanding of the nature of the QRS complex and its relation to myocardial excitation, there has developed the concept that the T wave represents a simple retreat of the wave of excitation, i.e., doublets of retreat.[6]

Without entering into a detailed examination of the two exact mechanisms, either explanation, if correct, should answer questions directed at the broader concept that the T wave is a disappearance phenomenon of an electric disturbance which in its invasion of the myocardium inscribes the QRS complex. One question which immediately rises is why the disappearance of the wave of excitation normally should involve a larger quantity of electricity than that involved in its entrance or invasion. This disparity can be demonstrated readily by comparing the sum of the areas under the QRS and T waves in the normal electrocardiogram taken from enough points over the body surface to encompass the heart completely.[6]† Other questions dealing with specific changes in the QRS without corresponding changes in the T wave, and vice versa, may be asked, and many will be unanswered. For instance, it is a common observation that the T waves increase in magnitude in hyperthyroidism without a corresponding increase in the QRS. If they were an expression of different phases of the same phenomenon, this hardly would seem likely.

*From the Department of Medicine, Northwestern University Medical School, Chicago.
Received for publication, June 15, 1942.

†The electrocardiograph machine is essentially a recording voltmeter which draws the minimum current from the electrical source. Thus the electrocardiogram is a potential-time tracing and the areas under the tracing are a measure of the quantities of electricity involved in each portion of the electrical cardiac cycle.

Another reason for this investigation deals not with the inadequacies of the current explanations but with the inherent electrodynamic characteristics of the T wave itself. A close study of the normal ventricular QRS-T complex shows that the QRS component and the T component are entirely different in form, duration of existence, and rate of change. The QRS tracing has a steep front and an equally steep decline, while the T-wave has a smoothly changing front, a more gradual rate of change, and persists for a much longer time. If the T-wave potential were dependent on cardiac muscular contraction, it would seem that the shape of the tracing could be correlated with a possible mechanism of development; that is to say, the shape would more nearly reflect its manner of production. Further, the greater quantity of electricity involved in the T wave could be explained, for in contracting the heart does work and is, therefore, potentially capable of producing electricity. Obviously, this idea would be untenable at once, were the T wave to be inscribed at any period in the cardiac cycle other than that of cardiac systole, or even begin before or persist after systole. This, however, is not the case, as can be shown by simultaneous intraventricular pressure and electrocardiographic recordings.

The association of the T-wave potential with myocardial activity is not new. Several interesting references to this possibility have appeared in the literature.[7, 8] One reference is to the specific idea to be outlined in the following paragraphs.[9]

In considering the conditions obtaining in the myocardium during systole which could convert mechanical energy into electrical, it is reasonable that those which are involved in a displacement phenomenon are the most likely subjects of investigation. On this hypothesis, then, the intramural displacement of fluids, both intercellular and capillary, suggests itself as a possible source by reason of the electrokinetic phenomen of streaming potentials.[10] The question is, will there by a sufficient pressure gradient along the intramural paths open to the blood, tissue fluid, and lymph to produce an appreciable electrokinetic effect?

From the equation for streaming potentials,

$$H = 8.48 \times 10^{-8} \frac{\zeta D P}{n K_S}$$

where H = Stream potential in millivolts
ζ = Potential of the double layer
n = Coefficient of viscosity
D = Dielectric constant
P = Pressure in cm. Hg
K_S = Specific conductivity in mhos

it is seen that for a given capillary system, the potential is directly *proportional* to the pressure, the dielectric constant, and the zeta potential; and inversely proportional to the viscosity and the *conductivity*. *A simple substitution* of the constants for whole blood at body temperature and an assumed value of 50 millivolts for the potential of the double layer will show, *however*, that the pressure will have to be high if the potential is to be of the *order of magnitude* necessary to produce the T wave. Calculations will readily show that ventricular

intramural stress can attain values of 40 cm. Hg throughout most of the wall and values of 60 cm. Hg at the base where the muscle fibers taper down to insert in the valve rings and intervalvular fibrosa.

As a preliminary test of the foregoing reasoning, dead hearts were perfused through the left coronary artery with various fluids (5 per cent glucose, 0.2 to 0.9 per cent sodium chloride, whole heparinized blood), and the potential across the anterior wall of the left ventricle was measured at various intramural pressures. At pressures between 20 and 40 cm. Hg significant potentials were developed, and the variations were in agreement with those predicted from the equation, namely, that the low conducting solutions produced the largest potential and the more conducting solutions the smaller potential, and that the potential was directly related to pressure. The potential developed with blood, which is the one of interest, was 0.5 millivolts at 40 cm. pressure. It should be noted here that the potentials developed varied considerably with the individual hearts and appeared to depend on the time dead, since higher values were obtained with the fresher material. These experiments, therefore, are not quantitative but qualitative, and the results are of value only in that they demonstrate that the internal architecture of the ventricular wall affords a capillary system which, when there is streamed through it a fluid with the comparatively high electrolyte concentration of whole blood, is capable of producing significant streaming potentials.

The question of the exact manner in which the blood and extracellular fluid may be displaced or squeezed about during ventricular systole to produce streaming potentials, cannot be answered dogmatically. The most logical sequence would seem to be a progressive "wringing out" of the myocardium as contraction progresses to a maximum. That blood, at least, is "wrung" out during systole is shown by the coronary (venous) outflow which occurs at this time in the cardiac cycle.[11, 12] For any given segment of myocardium, the sign of the developed potential would be negative to positive when progressing in the direction of the intermuscular fluid displacement.

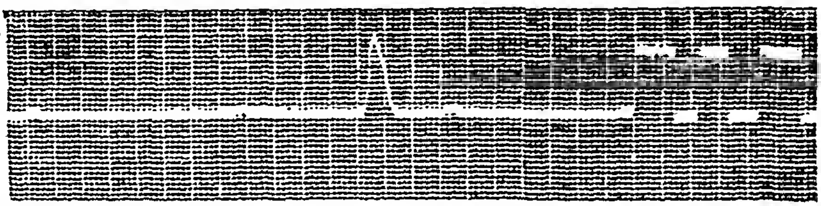
From the foregoing it can be postulated that regardless of the manner in which an intramyocardial pressure gradient is produced, whether by the actively contracting heart or by a force applied externally, just so long as it exists flow potentials should be developed. Thus, external energy applied to a heart rendered unresponsive to electrical and mechanical stimuli should produce a potential the characteristics of which are similar to those of the normal T wave as regards magnitude and rate of change. With this idea in mind, then, the following experiments were performed.

Series I.—Method. Dogs anesthetized with nembutal were given 40 c.c. of a 20 per cent solution of magnesium sulfate intravenously in order to decrease the irritability of the myocardium.[13] The thoracic cavity was then opened, care being taken to keep the field dry. The animal was connected through electrodes to the right foreleg and left hind leg to a string galvanometer electrocardiograph. The heart was then stimulated by means of a probe, and records were made of the resulting ectopic impulses. When stimulation ceased to produce an impulse, the heart was grasped in the hand which had been insulated by means of a rubber glove, and roughly bumped about the chest cavity to determine the amount of potential, if any, developed by such a procedure. With the electrocardiograph

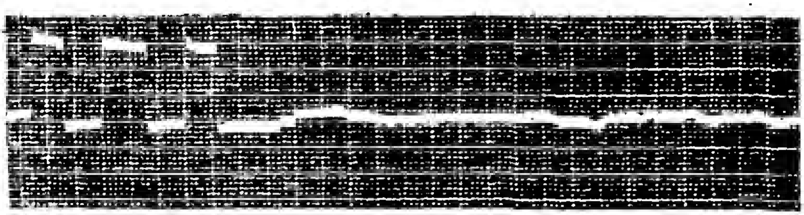
recording the heart was then forcibly constricted and suddenly released, or pressure was maintained for a period of several seconds.

Results. Cardiac stimulation produced ectopic impulses for a period varying up to twenty minutes after the chest cavity was opened. As will be seen from Fig. 1*A* a period was finally reached when stimulation produced an impulse without demonstrable contraction or T wave. Fig. 1*B* shows the result of movement of the heart and mediastinal structures. As will be seen, no significant potential was developed despite the fact that the contents of the chest were moved about vigorously, and the string was loosened to exaggerate the effect of any movement. Fig. 1*C* shows the result of constriction of the heart before irritability had entirely disappeared. Here it will be seen that there was a sudden production of a potential which gradually returned toward zero.

A.



B.



C.

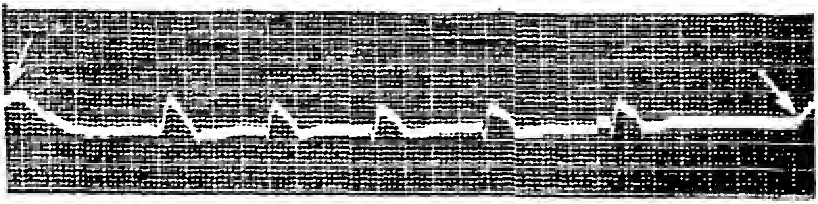


Fig. 1.—*A*, Stimulation of myocardium producing an impulse without demonstrable contraction or T wave. *B*, Vigorous movement of the heart and mediastinal structures. *C*, Arrows indicate application and relaxation of pressure. Note ectopic impulses occurring during this period.

At the same time several ectopic impulses arose from the yet irritable muscle, thus demonstrating that the potential that developed by squeezing the heart was independent of spontaneous impulses. Fig. 2*A* shows the result of sudden constriction of the heart with immediate relaxation, while Fig. 2*B* shows constriction with maintenance of pressure and the return of the potential to zero upon relaxation.

After irritability had ceased there was a period of from five to twelve minutes when the above results could be obtained at will. At the end of that time changes had occurred in the tissue which resulted in adventitious voltages due to development of local concentration cells, so that any movement of the thoracic structures would produce unlimited bizarre potentials.

Series II.—Method. These experiments were conducted to confirm previous observations regarding the time relationship of changes in intraventricular pressure to the T wave in the electrocardiogram. This was done by simultaneously recording on the electrocardiogram the pressure in the left ventricle and carotid artery.[14]

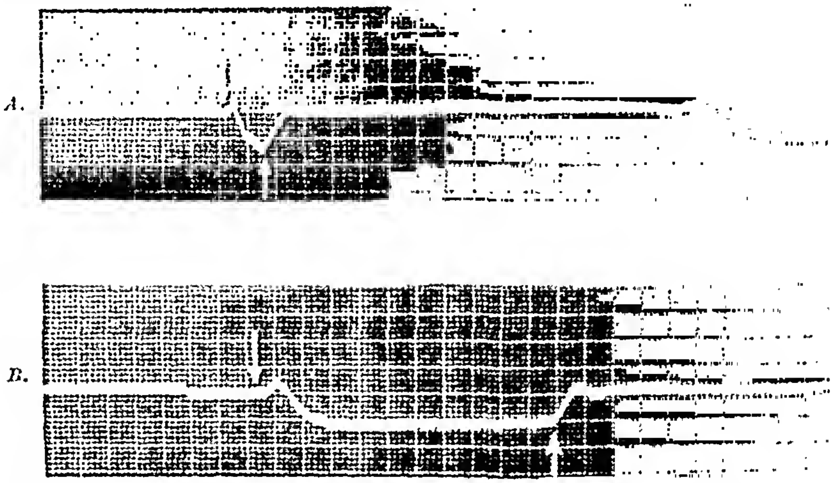


Fig. 2.—*A*, Constriction of heart with immediate relaxation. *B*, Constriction with maintenance of pressure and sudden relaxation.

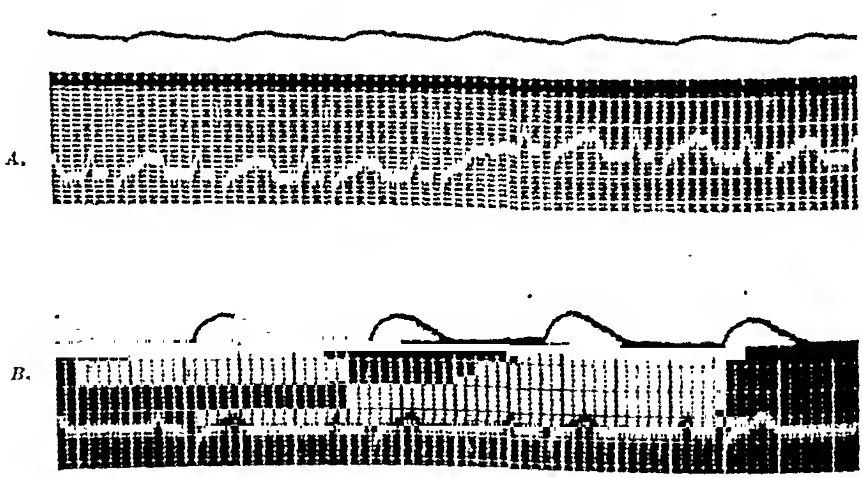


Fig. 3.—*A*, Simultaneous recording of carotid pressure and electrocardiogram. *B*, Left intraventricular pressure and electrocardiogram.

Results. Fig. 3*A* shows the correlation of increases in carotid pressure with the T wave and Fig. 3*B* shows changes in the left intraventricular pressure and the relationship of these changes to the T wave. It will be seen that in each case rise of pressure occurs at the same time as the T wave and that the latter is always confined within the time limits of the former.

SUMMARY AND CONCLUSIONS

The nature of the T wave in the electrocardiogram suggests that it is due to an electrokinetic cause and does not represent retreat electric disturbances.

The structure of the heart with its capillary bed is such that contraction could produce streaming potentials of the order of magnitude of the T wave. Pressure perfusion of the heart showed this to be entirely possible. Constriction of the unresponsive heart likewise produced this phenomenon. In addition it is shown that the T wave corresponds in time to the increase in pressure in the left ventricle. These observations suggest that contraction of the heart with its resultant streaming potentials is responsible for the T wave in the electrocardiogram.

REFERENCES

1. Adrian, E. D.: The Recovery Process of Excitable Tissues, J. Physiol. 55: 193, 1921.
2. Yoshida, H.: Zur Deutung des Elektrokardiogramms. I. Das Elektrogramm der Ventrikelspitze des Froschherzens, Ztschr. f. Biol. 84: 51, 1926.
3. Yoshida, H.: Zur Deutung des Elektrokardiogramms. II. Versuche am ganzen, frei aufgehängten Froschventrikel, Ztschr. f. Biol. 84: 453, 1926.
4. Lewis, Sir Thomas: The Mechanism and Graphic Representation of the Heart Beat, London, 1925, Shaw & Sons, Ltd.
5. Katz, L. N.: The Significance of the T-Wave in the Electrogram and Electrocardiogram, Physiol. Rev. 8: 447, 1928.
6. Craib, W. H.: The Electrocardiogram, Medical Research Council, Special Report Series, No. 147, London, 1930, H. M. Stationery Office.
7. Samojloff, A.: Weitere Beiträge zur Elektrophysiologie des Herzens, Arch. f. d. ges. Physiol. 135: 417, 1910.
8. Hill, A.: Analysis of the Normal T-Wave, Lancet 2: 979, 1930.
9. Krasno, M. R., Eyster, J. A. E., and Maaske, C. A.: The Nature of the T-Wave Potentials in the Tortoise Heart, Am. J. Physiol. 114: 119, 1935.
10. Abramson, H. A.: Electrokinetic Phenomena, New York, 1934, The Chemical Catalog Co.
11. Anrep, G. V., Cruikshank, E. W. H., Downing, A. C., and Rau, A. S.: The Coronary Circulation in Relation to the Heart Cycle, Heart 14: 111, 1927.
12. Anrep, G. V., and King, B.: The Significance of Diastolic and Systolic Blood Pressures for Maintenance of Coronary Circulation, J. Physiol. 64: 341, 1928.
13. Miller, J. Roscoe, and Van Dellen, T. R.: Electrocardiographic Changes Following the Intravenous Administration of Magnesium Sulfate, An Experimental Study on Dogs, J. Lab. & Clin. Med. 23: 914, 1938; 24: 840, 1939.
14. Hamilton, W. F., Brewer, G., Brotman, I.: Pressure Pulse Contours in the Intact Animal. I. Analytical Description of a New High Frequency Hypodermic Manometer, Am. J. Physiol. 107: 427, 1934.

Series II.—Method. These experiments were conducted to confirm previous observations regarding the time relationship of changes in intraventricular pressure to the T wave in the electrocardiogram. This was done by simultaneously recording on the electrocardiogram the pressure in the left ventricle and carotid artery.[11]

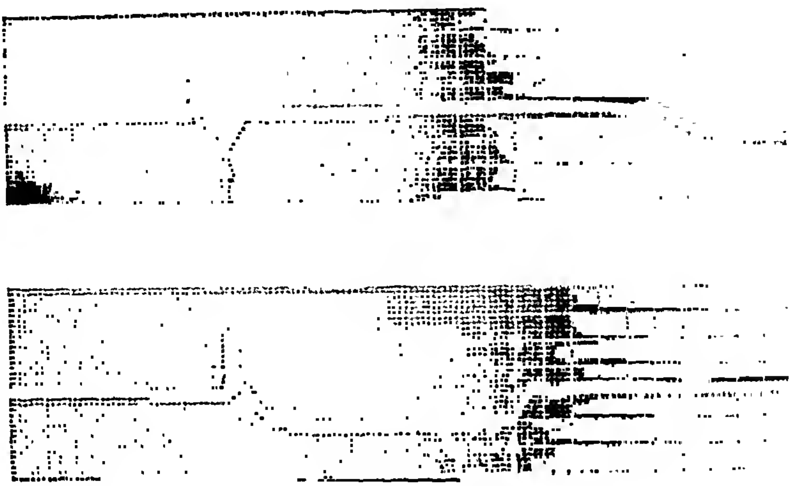


Fig. 2.—*A*, Constriction of heart with immediate relaxation. *B*, Constriction with maintenance of pressure and sudden relaxation.

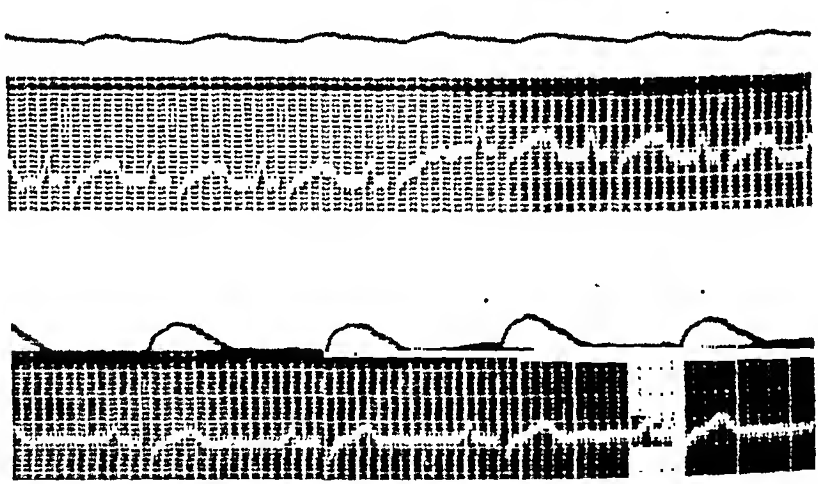


Fig. 3.—*A*, Simultaneous recording of carotid pressure and electrocardiogram. *B*, Left intraventricular pressure and electrocardiogram.

Results. Fig. 3*A* shows the correlation of increases in carotid pressure with the T wave and Fig. 3*B* shows changes in the left intraventricular pressure and the relationship of these changes to the T wave. It will be seen that in each case rise of pressure occurs at the same time as the T wave and that the latter is always confined within the time limits of the former.

SUMMARY AND CONCLUSIONS

The nature of the T wave in the electrocardiogram suggests that it is due to an electrokinetic cause and does not represent retreat electric disturbances.

The structure of the heart with its capillary bed is such that contraction could produce streaming potentials of the order of magnitude of the T wave. Pressure perfusion of the heart showed this to be entirely possible. Constriction of the unresponsive heart likewise produced this phenomenon. In addition it is shown that the T wave corresponds in time to the increase in pressure in the left ventricle. These observations suggest that contraction of the heart with its resultant streaming potentials is responsible for the T wave in the electrocardiogram.

REFERENCES

1. Adrian, E. D.: The Recovery Process of Excitable Tissues, J. Physiol. 55: 193, 1921.
2. Yoshida, H.: Zur Deutung des Elektrokardiogramms. I. Das Elektrogramm der Ventrikelspitze des Froschherzens, Ztschr. f. Biol. 84: 51, 1926.
3. Yoshida, H.: Zur Deutung des Elektrokardiogramms. II. Versuche am ganzen, frei aufgehängten Froschventrikel, Ztschr. f. Biol. 84: 453, 1926.
4. Lewis, Sir Thomas: The Mechanism and Graphic Representation of the Heart Beat, London, 1925, Shaw & Sons, Ltd.
5. Katz, L. N.: The Significance of the T-Wave in the Electrogram and Electrocardiogram, Physiol. Rev. 8: 447, 1928.
6. Craib, W. H.: The Electrocardiogram, Medical Research Council, Special Report Series, No. 147, London, 1930, H. M. Stationery Office.
7. Samojloff, A.: Weitere Beiträge zur Elektrophysiologie des Herzens, Arch. f. d. ges, Physiol. 135: 417, 1910.
8. Hill, A.: Analysis of the Normal T-Wave, Lancet 2: 979, 1939.
9. Krasno, M. R., Eyster, J. A. E., and Maaske, C. A.: The Nature of the T-Wave Potentials in the Tortoise Heart, Am. J. Physiol. 114: 119, 1935.
10. Abramson, H. A.: Electrokinetic Phenomena, New York, 1934, The Chemical Catalog Co.
11. Anrep, G. V., Cruikshank, E. W. H., Downing, A. C., and Rau, A. S.: The Coronary Circulation in Relation to the Heart Cycle, Heart 14: 111, 1927.
12. Anrep, G. V., and King, B.: The Significance of Diastolic and Systolic Blood Pressures for Maintenance of Coronary Circulation, J. Physiol. 64: 341, 1928.
13. Miller, J. Roscoe, and Van Dellen, T. R.: Electrocardiographic Changes Following the Intravenous Administration of Magnesium Sulfate, An Experimental Study on Dogs, J. Lab. & Clin. Med. 23: 914, 1938; 24: 840, 1939.
14. Hamilton, W. F., Brewer, G., Brotman, I.: Pressure Pulse Contours in the Intact Animal. I. Analytical Description of a New High Frequency Hypodermic Manometer, Am. J. Physiol. 107: 427, 1934.

CLINICAL CHEMISTRY

THE SIGNIFICANCE OF THE URINARY AMMONIA*

A. P. BRIGGS, AUGUSTA, GA.

CONSIDERABLE evidence has accumulated which indicates that it may be necessary to abandon one of our most time-honored concepts: the belief that secretion of ammonia by the kidneys serves in some way to maintain the acid-base balance of body fluids. Indeed, there is reason for suspecting that it has been carried along for a good many years by a combination of inertia and complacency.

The first note of dissension came in 1932 when it was observed that the acidosis of nephritis was not necessarily of the base deficit type, although the capacity for production of ammonia in this state was definitely subnormal. It was pointed out then that all observed facts relating to the secretion of ammonia were consistent with the hypothesis that ammonia production simply operates as a local protective mechanism to prevent excessive tubular acidity

Subsequently a number of special instances were observed in which the activity in production of ammonia was not that to be expected from the conventional theory but more in accord the dissenters' viewpoint.

This evidence will be reviewed briefly in the following sections and certain evidence provided by recent developments in the field of renal physiology will be presented.

CRUCIAL EXPERIMENTS

It should be obvious that the classical concept is based on circumstantial evidence which is perfectly compatible with either view, and that the whole question appears, superficially, to be merely a matter of words. Increased production of ammonia was observed to accompany increased excretion of acid, administered or metabolic. The ammonia was previously thought to be formed from neutral urea by the liver and perhaps by other tissues; secretion of a part of such acid in the form of ammonium salt was assumed to spare fixed alkali of the blood plasma. This assumption was entirely justified up to the time of the publication of the work of Nash and Benedict,[1] which indicated the kidney to be the organ of ammonia production. But the assumption, which has generally been made since then, that substitution of ammonia is part of the mechanism through which fixed base is reabsorbed from the glomerular fluid, and that it responds to requirements of the body for conservation of fixed base, is entirely gratui-

*From the Departments of Biochemistry and Internal Medicine of the University of Georgia Medical School, Augusta.

Received for publication, August 12, 1942.

tous—it is just as easy to suppose that production of ammonia by the tubules *follows* the reabsorption of fixed base as a result of irritation from rejected acid in the tubular fluid.

Since the acid-base balance of the blood and urine each deviates from the normal in the same direction, in most clinical and experimental states, it is impossible to tell from studies of such states whether fluctuations of ammonia are following changes in the plasma or renal tubules. There are available, however, a few studies in which the acid-base balance of the urine fluctuates in a direction opposite to that of the plasma. Such studies give crucial evidence.

EXPERIMENTS WITH SODIUM SALTS OF LOW THRESHOLD ACIDS

Hendrix and Sanders,[2] extending an experiment of Cushny,[3] observed that an acid urine may be formed following the administration of dibasic sodium phosphate or sodium hippurate, because the low threshold acids are not so actively reabsorbed by the renal tubules as is sodium. They also observed that production of ammonia was increased under these conditions in spite of the fact that the blood was "undoubtedly more alkaline." In my own studies with the sodium salt of another low threshold acid, sulfuric, similar changes were observed; the results were clear-cut following subcutaneous injection in the dog.[4]

The fluctuations of ammonia in these experiments, it should be observed, were parallel to the acid-base changes in the urine and were opposite to those of the plasma. They show clearly that production of urinary ammonia is not determined by the needs of the body for conservation of fixed base, for in each case the body was burdened with an excess of sodium. They strongly suggest that it is the acidity of the fluid flowing through the tubules which determines the level of ammonia production.

EXPERIMENTS WITH POTASSIUM CHLORIDE

Potassium chloride is a neutral salt which makes the urine alkaline for the reason that potassium, a low threshold base, is excreted much more rapidly than chloride. Consequently, the residual chloride which is left within the body causes an acid-base shift in the direction of acidity, similar to that which follows the administration of calcium chloride or hydrochloric acid.

Experiments with potassium chloride, administered orally or subcutaneously, have shown ammonia production to be immediately suppressed.[4] They make it clear that an excess of acid within the body is not an adequate stimulus for ammonia production. Again there is the suggestion that the acidity of the fluid flowing through the tubules is the determining factor.

THE INFLUENCE OF DIURESIS ON WASTE OF FIXED BASE AND ON AMMONIA PRODUCTION

The diuresis caused by drinking distilled water or by intravenous infusion of solutions of neutral substances, such as urea, results in the urinary waste of sodium bicarbonate. Presumably the rapid rate of flow through the tubules handicaps the mechanism of reabsorption of this vital plasma alkali. In the diuresis studies of Hendrix and Calvin[5] the plasma bicarbonate was lowered by as much as 15 per cent. But the ammonia mechanism fails to respond to the

base-deficit type of acidosis so produced,[6, 7] as it certainly should if it followed needs of the body for conservation of fixed base. This failure is in perfect harmony with the view which relates stimulation of ammonia production to increased tubular acidity. Such a diuresis does not produce an acid urine.

THE CONSERVATION OF FIXED BASE IN NEPHRITIS

There has been some confusion and controversy concerning the influence of nephritis on the renal mechanism for conserving fixed base. It is true that the capacity for production of ammonia is impaired with the destruction of many nephrons, and in such cases there is, occasionally, some evidence of base waste or depletion. The confusion is chiefly due to the obligation usually felt for incriminating the deficient ammonia mechanism as a causative factor—almost always it is possible to point to some other factor which might just as well be responsible.

Low levels of plasma fixed base are frequently encountered with the onset of uremia in nephritis, but vomiting is also frequent at this stage and is a very potent cause for such low levels. In a selected group of patients[8] who had advanced nephritis but who were not vomiting, the plasma fixed base values fell between 145 and 152 milliequivalents per liter; normal values with the same technique fell between 148 and 155. This slight difference, while hardly significant, might well be a diuresis effect. These patients had a polyuria and relatively few surviving nephrons. Consequently the rapid rate of tubular flow should have offered some handicap to tubular reabsorption.

If depressed levels of plasma fixed base result from the excretion of acid which would normally be carried off by ammonia, then prolonged administration of acid should have a very pronounced effect in lowering these levels in nephritis. Such, however, is not the case. In the studies of Linder,[9] Gamble, Blackfan, and Hamilton,[10] as well as that of my own,[8] there was no significant change in the level of plasma fixed base, in either normal or nephritic subjects as a result of heavy dosage of acid. This is one of the most important observations relating to the question of base waste in nephritis.

In certain instances an excessive amount of fixed base has been found to be excreted into the urine of nephritic patients as a result of acid administration. The urinary excretion of the various acids and bases was determined in each of the above-mentioned studies of acid ingestion. Gamble and co-workers observed that excretion of extra fixed base by their nephritic patient was excessive in comparison with their normal controls. However, this was a patient with an edematous type of nephritis, and so the significance is uncertain—delivery of edema fluid from any cause should result in the elimination of extra fixed base. Linder's study gave complete results on two nephritic subjects without edema. One excreted an excessive amount of fixed base, while the other did not. In my own study one of three nephritic subjects excreted an excessive quantity of fixed base, but this was accompanied by a conspicuous diuresis—twenty-four-hour urine volumes greater than 3,000 c.c.

The evidence for impaired conservation of fixed base in nephritis is certainly inconclusive, and the incrimination of the defective ammonia mechanism for such evidence as is available seems quite unnecessary.

THE EVIDENCE OF RECENT DEVELOPMENTS IN RENAL PHYSIOLOGY

The results of much critical study[11] tend to support the essentials of the "modern theory of urine formation," as elaborated by Cushny. The glomerular fluid seems to be a true filtrate which is formed, in an adult, at the rate of about 120 c.c. per minute. It also appears that the resultant fluid formed by reabsorption in the various compartments of the tubule system must be of fairly constant composition, especially so in regard to the constituents of the basic elements; otherwise the levels of these constituents in the plasma would not be maintained within their observed narrow ranges. The tubules do, however, secrete a few substances. Perhaps the most important of these is ammonia, the secretion of which in the amphibian tubule has lately been reported by Walker.[12]

QUANTITATIVE RELATIONS BETWEEN SECRETION OF AMMONIA AND REABSORPTION OF FIXED BASE

The notion that secretion of ammonia is necessary for reabsorption of fixed base probably rests on the failure to bear in mind the quantitative relations of the two processes. With glomerular filtration at the rate of approximately 120 c.c. per minute, and with a fixed base concentration of about 150 meq. per liter, something like 1,080 meq. per hour of fixed base is filtered. Consequently, with the urinary excretion of fixed base at levels varying from 2 to 20 meq. per hour, the magnitude of fixed base reabsorption is seen to be at the enormous level of more than 1,000 meq. per hour. Under ordinary conditions the output of ammonia amounts to something less than 3 meq. per hour. It is, therefore, perfectly clear, when the magnitudes of these two processes are contrasted, that reabsorption of fixed base does not depend on substitution of ammonia. The highest levels of ammonia production, such as those observed in diabetic acidosis, are of the order of 25 meq. per hour, and so even these are hopelessly inadequate in accounting for fixed base reabsorption. The comparison is somewhat improved by making an additional assumption, which would bestow on the renal epithelium the capacity for differentiating between the base opposed by bicarbonate and the base opposed by chloride and other strong acid; ammonia to be substituted only for base opposed by bicarbonate. But this fraction of the total fixed base amounts to nearly 200 meq. per hour, which is again far greater than the maximum observed levels of ammonia production.

WORK ON THE ISOLATED NEPHRON OF AMPHIBIA

This work has been done on material aspirated directly from various levels of the tubule. In the proximal tubule there is no secretion of ammonia,[12] but glucose is reabsorbed and presumably along with it a considerable amount of water and salt.[13] The fixed base of this salt is therefore reabsorbed without substitution of ammonia. Actually ammonia was not found to appear before the distal half of the distal convoluted tubule, but chloride was actively reabsorbed by the first part of the distal tubule, and without change in pH,[14] so it must have been accompanied by an equivalent quantity of fixed base. Reabsorption of additional fixed base is thus accomplished without substitution of ammonia.

Acidification of the urine was observed to occur in a narrow zone near the middle of the distal tubule. The process of acidification was thought to depend on reabsorption of base bicarbonate. Since it was at this zone of acidification that secretion of ammonia was found to be initiated by Walker,[12] it is of great interest to speculate on the relation of the two processes. It is evident that ammonia is not substituted for all the base bicarbonate reabsorbed in this zone, for if so there would be no acidification. The manner in which ammonia is usually thought to operate is by interaction with salts of the glomerular fluid according to the reversible equilibrium:

$$NH_4HCO_3 + BAc \rightleftharpoons NH_4Ac + BHCO_3$$

According to this scheme, base bicarbonate would be reabsorbed and the acid radical would be carried off into the urine as ammonium salt.[11] But ammonia has been observed to be secreted only in the presence of acid.[12] Consequently, such a reversible equilibrium could never get anywhere in either direction because of the vigorous interaction of ammonia with acid, already liberated according to the scheme:

$$NH_4HCO_3 + HAc \rightarrow NH_4Ac + H_2CO_3$$

This reaction would very rapidly be carried to completion because of the slightly dissociated character of H_2CO_3.

Since ammonia reacts with acid which results from reabsorption of base bicarbonate, it might be said that the arrival of ammonia, unfortunately, is too late for the rescue. The simultaneous appearance of ammonia and acid formation is, to say the least, suggestive that secretion of ammonia operates as a local protective mechanism to prevent excessive acidity.

STUDIES OF THE MAMMALIAN NEPHRON

Preliminary reports from two separate laboratories[15, 16] each indicate that reabsorption of bicarbonate, that is acid production, may occur in the proximal tubule of the mammalian nephron. If this is true, it means there can only be a limited field of action for interchange of ions between ammonium bicarbonate and salts of the glomerular filtrate—even if secretion of ammonia should be found to occur at a level above the zone of acidification. If, as in Amphibia, secretion of ammonia is initiated at the zone of acidification, then the interpretation will be that indicated above; the classical concept relating secretion of ammonia to conservation of fixed base will have to be radically revised.

SUMMARY AND CONCLUSIONS

Crucial experiments show that ammonia production can, at will, be made to fluctuate in a direction opposite to that of any given acid-base change in the body. In all such experiments ammonia production fluctuates in the same direction as the acid-base change of the urine.

With advanced nephritis, in spite of curtailed production of ammonia, there is no clear-cut evidence of impaired conservation of fixed base. The evidence which is available does not necessarily incriminate the ammonia mechanism. Other factors, such as vomiting or diuresis, are usually present in instances of defective base conservation.

Examination of fluid taken from various levels of the amphibian nephron reveals that secretion of ammonia *follows* reabsorption of fixed base; and that ammonia is only secreted in the presence of acid, which precludes an interchange of ions in the reversible equilibrium usually assumed to be involved in the substitution of ammonia for fixed base.

It, therefore, seems improbable that the renal ammonia mechanism has anything to do with the regulation of the acid-base balance of the body. The evidence at hand appears to be more consistent with the hypothesis that secretion of ammonia is stimulated by, and serves to neutralize, the acid residue left in the tubules by reabsorption of the alkaline threshold moiety.

REFERENCES

1. Nash, T. P., and Benedict, S. R.: The Ammonia Content of the Blood, and Its Bearing on the Mechanism of Acid Neutralization in the Animal Organism, J. Biol. Chem. 48: 463, 1921.
2. Hendrix, B. M., and Sanders, J. P.: The Effect of Injections of Sodium Phosphates and Sodium Hippurate Upon the Excretion of Acid and Ammonia by the Kidney, J. Biol. Chem. 58: 503, 1923.
3. Cushny, A. R.: The Secretion of the Urine, London, 1917, Longmans, Green & Co.
4. Briggs, A. P.: Excretion of Ammonia and Neutrality Regulation, J. Biol. Chem. 104: 231, 1934.
5. Hendrix, B. M., and Calvin, D. B.: The Loss of Bases in Diuresis and Its Effect Upon the Alkali Reserve of the Blood, J. Biol. Chem. 65: 197, 1925.
6. Carr, A. D.: The Effect of Water Diuresis on the Elimination of Certain Urinary Constituents, J. Pharmacol. & Exper. Therap. 18: 221, 1921.
7. Barnett, G. D., and Blume, F. E.: Alkaline Tides, J. Clin. Investigation 17: 152, 1938.
8. Briggs, A. P.: The Acidosis of Nephritis; Its Clinical Significance, Arch. Int. Med. 49: 56, 1932.
9. Linder, G. C.: The Effect of Mineral Acid on Acid-Base Regulation in Health and in Nephritis, Quart. J. Med. 20: 285, 1927.
10. Gamble, J. L., Blackfan, K. D., and Hamilton, B.: A Study of the Diuretic Action of Acid Producing Salts, J. Clin. Investigation 1: 359, 1925.
11. Smith, Homer, W.: The Physiology of the Kidney, New York, 1937, Oxford Univ. Press.
12. Walker, A. M.: Ammonia Formation in the Amphibian Kidney, Am. J. Physiol. 131: 187, 1940.
13. Walker, A. M., Hudson, C. L., Findley, I., Jr., and Richards, A. N.: The Total Molecular Concentration and the Chloride Concentration of Fluid From Different Segments of the Renal Tubule of Amphibia, Am. J. Physiol. 118: 121, 1937.
14. Montgomery, H., and Pierce, J. A.: The Site of Acidification of the Urine Within the Renal Tubule in Amphibia, Am. J. Physiol. 118: 144, 1936.
15. Walker, A. M., and Bott, Phyllis, A., Oliver, Jean, and MacDowell, Muriel C.: The Collection and Analysis of Fluid From Single Nephrons of the Mammalian Kidney, Am. J. Physiol. 134: 580, 1941.
16. Ellinger, P.: Site of Acidification of Urine in Frog's and Rat's Kidney, Quart. J. Exper. Physiol. 30: 205, 1940. (Brit. Chem. & Physiol. Ab. A, III.—16, January, 1941.)

BROMSULPHALEIN RETENTION IN LOW-GRADE CHRONIC ILLNESS

MAJOR M. H. STILES, M.C., U. S. ARMY, M. T. STILES, AND A. McM. KOLB
PHILADELPHIA, PA.

LIVER dysfunction has been reported associated with a variety of chronic or recurrent conditions; e.g., allergy,[1-4] "rheumatism" and arthritis,[5-11] peptic ulcer,[12] glaucoma,[13] Parkinsonism,[4] thyrotoxicosis,[14-17] rheumatic fever,[18] various infections and bacterial intoxication.[4, 12, 19-23] Because of these reports liver function studies were made on a group of patients with low-grade chronic illness probably secondary to chronic infection.

The clinical condition, sedimentation rate, and nonfilament-filament ratio,[24, 25] incidence of probable pathogenic streptococci and staphylococci[26] and distribution of metabolic rates[27] of this group have been reported. One hundred and twelve ambulant patients, varying in age from 15 to 82, were included. They were classified according to age, sex and the severity of objective symptoms.

In the choice of a method for determining liver function the bromsulphalein test was considered favorably, since it could be performed in an office on ambulant patients, it did not consume much of the patient's time, and it was considered by many to be the most generally satisfactory test in the absence of jaundice.[28-31] However, while some[32, 33] felt that the usual bromsulphalein test was very sensitive and would indicate early, and perhaps even temporary liver dysfunction, others[5, 34, 35] felt that it was necessary to devise an even more delicate test for use in mild cases. Macdonald,[36, 37] who studied this question intensively, pointed out that because of the great liver reserve a normal result with the usual bromsulphalein test did not indicate an absence of hepatic damage, although an abnormal retention almost definitely meant a diseased liver. Studying liver reserve in the search for a test which would detect minor grades of impairment, he developed a technique by means of which the blood could be examined at two-minute intervals following the injection of bromsulphalein. With this method he observed that the rate of bromsulphalein removal from the blood of normal persons followed a definite curve, and that disease of the liver produced not only a retention of bromsulphalein beyond the usual test period, but also a change in the type of curve. As a result of his observations Macdonald concluded that a quantity of bromsulphalein equivalent to 5 mg. per kilogram of body weight was necessary to test fully the liver reserve, that the normal undamaged liver would completely free the blood of this quantity of bromsulphalein in twenty-five minutes, and that the maximum information about liver function would only be obtained if the blood level of bromsulphalein were determined every five minutes. In a recent

Received for publication, July 11, 1942.

evaluation of liver function tests Mateer and his colleagues[38] found Macdonald's serial bromsulphalein test sensitive and reliable, even though the 2 mg. per kilogram dose was mainly used. Suggestions for fractional tests of liver function have also been made by White, Deutsch, and Maddock,[39] and by Deutsch.[40]

TABLE I

STATISTICAL ANALYSIS OF BROMSULPHALEIN RETENTION DISTRIBUTED ACCORDING TO SEX, AGE, SEVERITY OF SYMPTOMS AND SEDIMENTATION RATE

GROUP	NO.	RANGE	MEAN	±	S.E.	% 0	% 5-20	% 20+
All patients	112	0-90	18.93	±	1.63	14.3	56.3	29.3
Sex								
Male	45	0-70	19.33	±	2.45	15.6	51.1	33.3
Female	67	0-90	18.66	±	2.18	13.4	59.7	26.9
Age								
Up to 20	9	0-60	18.33	±	5.84	11.1	66.6	22.2
21-40	27	0-50	12.78	±	2.4	25.9	52.0	22.1
41-60	49	0-90	17.96	±	2.54	12.2	65.4	22.4
61+	27	0-70	27.04	±	3.4	7.4	40.8	51.8
Symptoms								
Mild	48	0-30	10.0	±	1.19	22.9	70.9	6.2
Moderate	47	0-50	18.94	±	1.83	10.6	53.2	36.2
Severe	17	5-90	44.71	±	5.25	0.0	23.5	76.5
Sedimentation rate in mm. (Westergren)								
1-10	59	0-50	14.75	±	1.71	20.4	57.5	22.1
11-20	28	0-90	20.71	±	3.89	10.7	57.1	32.2
21-30	13	0-60	22.31	±	4.68	7.7	53.9	38.4
31+	8	5-70	32.50	+	7.39	0.0	37.5	62.5

No. = Number of patients in group. Range = range of bromsulphalein retention in units. S.E. = Standard error. % 0 = per cent of patients in group with no bromsulphalein retention. % 5-20 = per cent with 5 to 20 units retention. % 20 + = per cent with more than 20 units retention.

In the present study Macdonald's normal criterion of complete elimination in twenty-five minutes of a dose of bromsulphalein equal to 5 mg. per kilogram body weight was adopted. The estimated quantity of bromsulphalein solution diluted with an equal quantity of physiologic salt solution was slowly injected with a No. 26 gauge needle. For simplicity usually only a single specimen of blood was withdrawn twenty-five minutes later. The use of larger needles and of full strength bromsulphalein solution in early tests was occasionally followed by thrombophlebitis, which in one instance was moderately severe and resulted in several days' incapacitation.

RESULTS

A statistical analysis of the results of the bromsulphalein tests is presented in Table I. Of the 112 patients only 16 showed no retention of bromsulphalein at the end of twenty-five minutes. If the blood had been examined at five-minute intervals throughout the test period, it is probable that even some of these would have shown abnormal curves.[41] Somewhat more than half of the total number showed bromsulphalein retention of from 5 to 20 units. The remaining patients showed higher degrees of retention, the highest being 90 units. The mean value for the entire group was 18.9. The frequency distribution of the tests is illustrated in Fig. 1.

When the observations were distributed according to sex, very little difference was found, the mean bromsulphalein retention being 19.3 units for males and 18.7 units for females (Fig. 2). When the observations were distributed according to age, greater differences were noted (Fig. 2). The mean bromsulphalein retention in units for patients up to 20 was 18.3; 21 to 40, 12.8; 41 to 60, 17.0; and 61 and over, 27.0. The mean rates for patients 21 to 40, and for those 61 and over, varied significantly from the mean of the entire group.

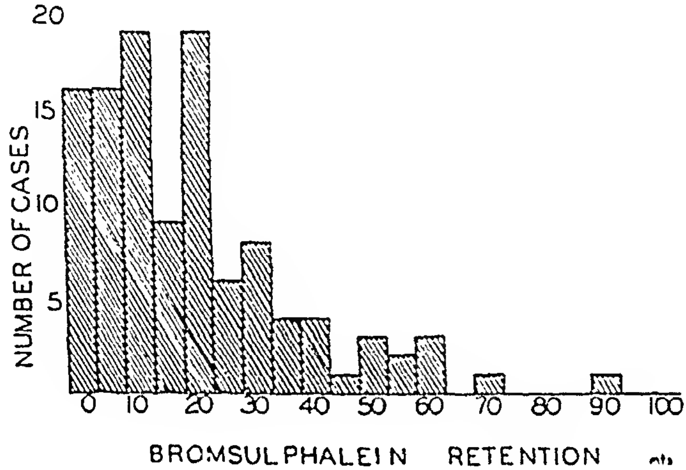


Fig. 1.—Frequency distribution of bromsulphalein retention in 112 patients with low-grade chronic illness.

When the observations were distributed according to the severity of symptoms (Fig. 3), greater differences appeared. The mean bromsulphalein retention in patients with mild symptoms was 10 units; in patients with moderately severe symptoms, 18.9 units; and in patients with severe symptoms, 44.7 units. Of the patients with mild symptoms 22.9 per cent showed no retention of bromsulphalein and only 6.2 per cent showed retention of more than 20 units. Of those with severe symptoms all showed some retention and 76.5 per cent showed retention of more than 20 units.

Since it has been suggested that there may be a relation between liver function and the sedimentation rate of erythrocytes,[42] the bromsulphalein retention and the sedimentation rate (Westergren) were compared (Fig. 4). The mean bromsulphalein retention for patients with sedimentation rates, ranging from 1 to 10 mm., was 14.75 units; 11 to 20 mm., 20.7 units; 21 to 30 mm., 22.3 units; and over 30 mm., 32.5 units. Yet, while in general higher sedimentation rates were associated with higher degrees of bromsulphalein retention, the association was far from complete. For example, in patients with sedimentation rates up to 10 mm. the degree of bromsulphalein retention varied from 0 to 50 units, and in those with sedimentation rates of 31 mm. or higher it varied from 5 to 70 units. The lack of a definite linear relation between sedimentation rate and bromsulphalein retention is illustrated by the scatter diagram in Fig. 5.

The differences between the mean bromsulphalein retention rates of patients with mild, moderately severe, and severe symptoms were all statistically significant, as were also the variations in relation to changes in sedimentation rate.

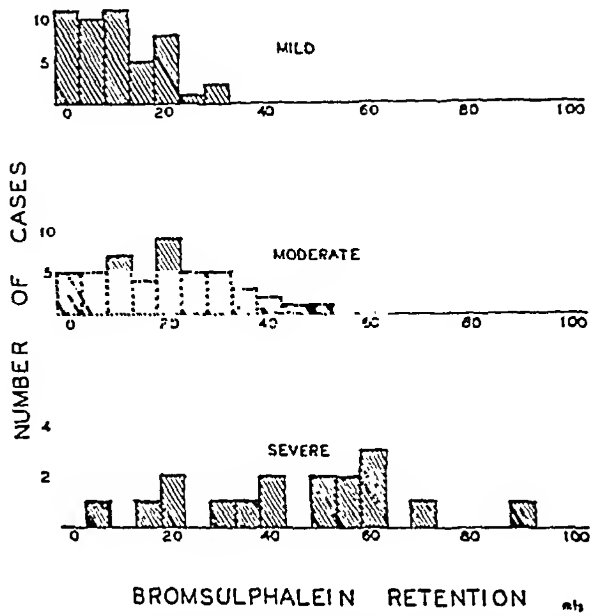


Fig. 2.—Bromsulphalein retention distributed according to sex and age.

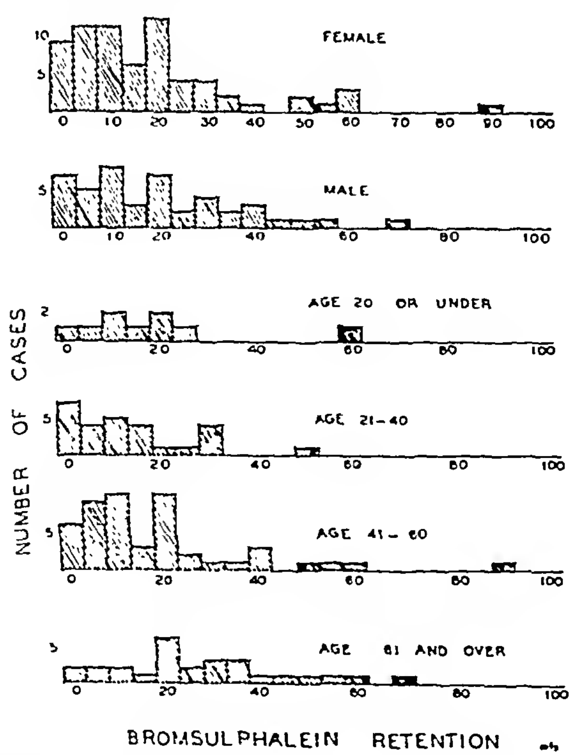


Fig. 3.—Bromsulphalein retention distributed according to severity of general symptoms.

COMMENT

The high incidence of impaired liver function in persons with chronic illness should not cause surprise if the reports of earlier writers are recalled. Rehfuss[43] has stated that biliary tract disturbance is probably present in one half of all persons past the age of 40. MacLagan and Rundle[17] stated that of 41 patients with hyperthyroidism, 30 showed definite impairment of hepatic function and the remaining 11 were border line. Shaffer,[16] in examining the livers of persons who died accidental deaths, found that 31 of 50 showed fatty infiltration, and 11 of 50 showed chronic localized interstitial hepatitis. Rosenberg[44] used the cephalin-cholesterol flocculation test in studying a group of 155 persons of whom 83 had clinical evidence of liver disease. He found liver impairment in practically all of those with clinical liver disease and in 43 of

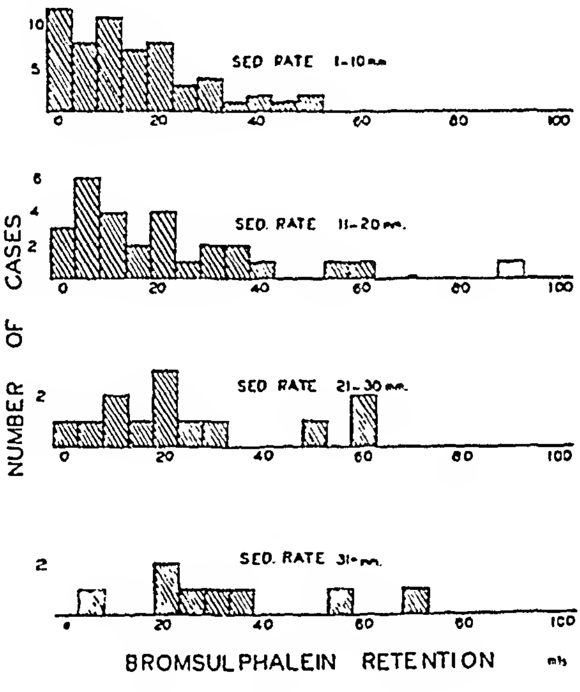


Fig. 4.—Frequency distribution of bromsulphalein retention in relation to sedimentation rate.

the 72 in whom hepatic disease was not suspected. He concluded that mild and subclinical disease of the liver occurs more frequently than is generally appreciated, and unless specifically sought may elude recognition. Apparently liver disturbance is frequent even in early life, since Londe and Probstein[45] found evidence of disturbed liver function in two children whose only departure from health had been recent upper respiratory infections, and in 9 of 13 children with histories of repeated colds. These findings are consistent with the observations made in the present study, where 8 of 9 children and adolescents showed some degree of bromsulphalein retention.

While the variations in bromsulphalein retention in relation to age are statistically significant, their actual significance is somewhat impaired by the high proportion of patients with mild symptoms in the age group 21 to 40, and

the high proportion with severe symptoms in the age group 61 and over. It may be significant that in the series of studies which have been made on patients with low-grade chronic illness,[24, 26, 27] the group from 21 to 30 years of age showed fewer deviations from the normal than did any other group.

The variations in bromsulphalein retention in relation to severity of symptoms also are consistent with the observations made in other studies of low-grade chronic illness. Patients with mild symptoms showed the least deviation from normal in the sedimentation rate,[24] the nonfilament-filament ratio,[24] the incidence of pathogenic streptococci,[26] and in the basal metabolic rate;[27] and persons with severe symptoms showed the greatest deviation from normal in all of these tests. Rawls, Weiss, and Collins[8] noted that liver dysfunction occurred with greater frequency in patients with severe than in those with mild rheumatoid arthritis.

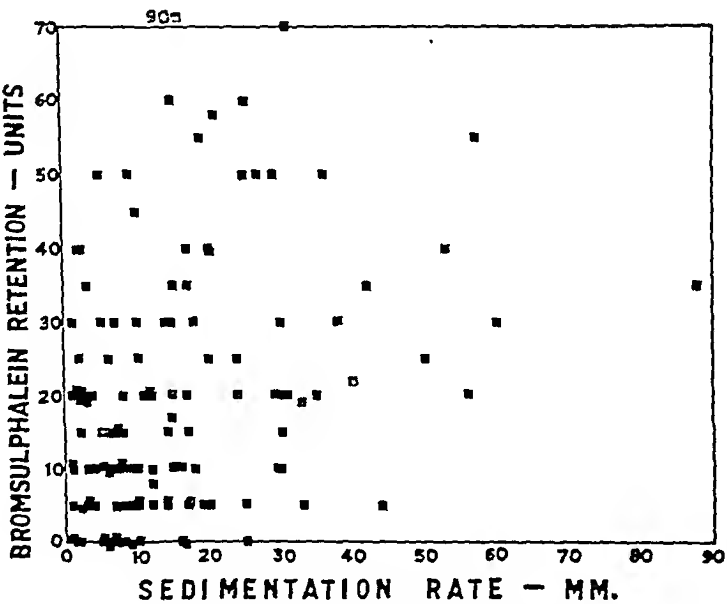


Fig. 5.—Relation of bromsulphalein retention (units) and sedimentation rate (mm. in one hour, Westergren) in individual patients.

While patients with high sedimentation rates had significantly greater bromsulphalein retention than did those with low sedimentation rates, it is possible that abnormal results with both tests are related more to the severity of illness than to each other. In view of the high degree of bromsulphalein retention occasionally associated with a practically normal sedimentation rate, and the high sedimentation rates associated with little bromsulphalein retention, there does not seem to be a direct relation between the two tests. It is possible, of course, that some other test of liver function might give results more nearly parallel to the sedimentation rate. It seems more probable, however, that, as Wintrobe[46] has said of the sedimentation rate, neither the sedimentation rate nor the bromsulphalein retention should be regarded as a precise, quantitative measurement, but should be accepted as a general indication of somatic or hepatic disturbance.

SUMMARY

Studies of bromsulphalein retention were made on 112 patients with low-grade chronic illness, using a dose of 5 mg. per kilogram of body weight.

On the basis of Macdonald's criterion of normal excretion all but 16 showed evidence of liver dysfunction. Of the 96 with dysfunction 33 showed more than 20 units retention of bromsulphalein.

The mean bromsulphalein retention of males differed little from that of females. The mean retention of patients aged 21 to 40 years was less than the general average and that of patients over 60 was greater, but the actual significance is uncertain.

When the mean bromsulphalein retention of patients with mild general symptoms was compared with the mean retention of patients with moderately severe and severe symptoms, statistically significant differences were found, the respective values being 10.0, 18.9 and 44.7 units.

While patients with high sedimentation rates tended to have a high degree of bromsulphalein retention, there were a number with high rates who had little retention, and a number with low rates who had considerable retention.

CONCLUSIONS

Impairment of liver function is found in a considerable proportion of patients with low-grade chronic illness.

The degree of impairment varies directly with the severity of symptoms.

REFERENCES

1. Shay, H., Gersohn-Cohen, J., and Fels, S. S.: The Factor of Occult Hepatic and Biliary Tract Disease in Some Cases of Allergy, Am. J. Digest. Dis. 6: 335, 1939.
2. Schmengler, F. E.: Hepatic Cirrhosis and Hepatargy on Allergic Basis, Klin. Wchnschr. 19: 1155, 1940.
3. Coke, F., and Coke, H.: Asthma, Baltimore, 1939, Williams & Wilkins Co.
4. Todd, A. T.: Treatment of Some Chronic and Incurable Diseases, Bristol, 1937, John Wright & Sons, Ltd.
5. Hench, P. S., Bauer, W., Dawson, M. H., Hall, F., Holbrook, W. P., Key, J. A., and McEwen, C.: The Problem of Rheumatism and Arthritis, Ann. Int. Med. 14: 1383, 1941.
6. Burbank, R.: An Outline of the Aetiology, Pathology and Treatment of Arthritis, Tri-State M. J. 12: 2355, 1939.
7. Collins, V. L.: The Relation of Liver and Gallbladder Disease to Arthritis, Rev. Gastroenterol. 6: 344, 1939.
8. Rawls, W. B., Weiss, S., and Collins, V. L.: Liver Function in Rheumatoid Arthritis, Ann. Int. Med. 10: 1021, 1937.
9. Rawls, W. B., Weiss, S., and Collins, V. L.: Liver Function in Rheumatoid Arthritis, Ann. Int. Med. 12: 1455, 1939.
10. Peers, R. S.: Gallbladder: Its Relation to Chronic Arthritis of Uncertain Etiology, California & West. Med. 51: 374, 1939.
11. Andrews, K. R., and Muether, R. O.: Effect of Diet on Arterial and Venous Glucose Tolerance Curve in Rheumatoid Arthritis, J. Lab. & Clin. Med. 26: 675, 1941.
12. Weir, J. F.: Hepatitis: Some Forms Not Commonly Recognized, J. A. M. A. 111: 1356, 1938.
13. Schmelzer, H.: Examination of Hepatic Function in Patients With Glaucoma, Arch. f. Ophth. 141: 266, 1939.
14. Haines, S. F., Magath, T. B., and Power, M. H.: The Hippuric Acid Test in Hyperthyroidism, Ann. Int. Med. 14: 1225, 1941.
15. Lichtmann, S. S.: Liver Function in Hyperthyroidism, With Special Reference to the Galactose Tolerance Test, Ann. Int. Med. 14: 1199, 1941.
16. Shaffer, J. M.: Disease of Liver in Hyperthyroidism, Arch. Path. 29: 20, 1940.
17. MacLagan, N. F., and Rundle, F. F.: Liver Function in Thyrotoxicosis, Quart. J. Med. 9: 215, 1940.

18. Lichtmann, S. S.: Cinchophen Oxidization Test of the Function of the Hepatic Cells, Arch. Int. Med. 48: 98, 1931.
19. Schottmuller, H.: Hepatitis and Cholecystitis, Specific Toxic Complication of Scarlet Fever, Klin. Wchnschr. 10: 17, 1931.
20. Steidl, J., and Heise, F. H.: Studies of Liver Function in Advanced Pulmonary Tuberculosis, Am. J. M. Sc. 186: 631, 1933.
21. Cates, H. B.: Relation of Liver Function to Cirrhosis of Liver and to Alcoholism, Arch. Int. Med. 67: 383, 1941.
22. Portis, S. A.: Hepatitis: Etiology, Diagnosis and Treatment, New Orleans M. & S. J. 92: 415, 1940.
23. McNee, J. W.: Hepatitis, Edinburgh M. J. 46: 305, 1939.
24. Stiles, M. H.: Sedimentation Rate and Nonfilament-Filament Ratio in Low Grade Chronic Illness, Arch. Int. Med. 63: 664, 1939.
25. Stiles, M. H.: Changes in Sedimentation Rate and Nonfilament-Filament Ratio Following Clinical Improvement in Patients With Low Grade Chronic Illness, J. Lab. & Clin. Med. 26: 621, 1941.
26. Stiles, M. H., and Chapman, G. H.: Probable Pathogenic Streptococci and Staphylococci in Chronic Low Grade Illness, Arch. Otolaryng. 31: 458, 1940.
27. Stiles, M. H.: Basal Metabolic Rate in Low Grade Chronic Illness. A Statistical Analysis of 166 Cases, Am. J. Clin. Path. 11: 871, 1941.
28. Gerendasy, J.: An Evaluation of Liver Function Tests, Rev. Gastroenterol. 7: 186, 1940.
29. Newell, T. E., and Hoerner, M. T.: The Diagnosis and Treatment of Jaundice, Ohio State M. J. 36: 365, 1940.
30. Snell, A. M., and Magath, T. B.: The Use and Interpretation of Tests for Liver Function, J. A. M. A. 110: 167, 1938.
31. Editorial: The Effect of Anesthetics on Hepatic Function, J. A. M. A. 110: 1755, 1938.
32. Israel, N. L., and Reinhold, J. G.: Detection of Cirrhosis and Other Diseases of the Liver by Laboratory Tests, J. Lab. & Clin. Med. 23: 588, 1938.
33. DeLor, C. J., and Reinhart, H. L.: An Analysis of the Hippuric Acid, Galactose Tolerance, Bromsulphalein and Prothrombin Tests in 381 Cases, Am. J. Clin. Path. 10: 617, 1940.
34. Morrison, L. M., and Swalm, W. A.: New Physiologic Test for Liver Function: Preliminary Report, Rev. Gastroenterol 7: 269, 1940.
35. Rosenberg, D. H., and Soskin S.: The Azorubin S Test for Liver Function: An Evaluation, With a Comparative Study of the Bromsulphalein and Hippuric Acid Tests, Ann. Int. Med. 13: 1645, 1940.
36. Macdonald, D.: Some Observations on Disappearance of Bromsulphalein Dye From the Blood: Its Relation to Liver Function (Preliminary Report), Canad. M. A. J. 39: 556, 1938.
37. Macdonald, D.: A Practical and Clinical Test for Liver Reserve, Surg., Gynec. & Obst. 69: 70, 1939.
38. Mateer, J. G., Baltz, J. I., Marion, D. F., Hollands, R. A., and Yagle, E. M.: A Comparative Evaluation of the Newer Liver Function Tests, Am. J. Digest. Dis. 9: 13, 1942.
39. White, F. W., Deutsch, E., and Maddock, S.: The Comparative Value of Serial Hippuric Acid Excretion, Am. J. Digest. Dis. 6: 603, 1939.
40. Deutsch, E.: A Fractional Bromsulphalein Test to Determine Liver Damage in the Nonjaundiced Patient, New England J. Med. 225: 171, 1941.
41. Macdonald, Dean: Personal communication.
42. Lichtmann, S. S.: The Influence of Intravenous Glucose Injections on Abnormal Erythrocyte Sedimentation Speed in Relation to Activity of Infection, Ann. Int. Med. 13: 1297, 1940.
43. Rehfuss, M. E.: The Management of the Gallbladder Patient, Pennsylvania M. J. 42: 1335, 1939.
44. Rosenberg, D. H.: Cephalin-Cholesterol Flocculation Test in Cases of Disease of Liver, With Special Reference to Diagnosis of Mild and Unsuspected Forms, Arch. Surg. 43: 231, 1941.
45. Londe, S., and Probstein, J. G.: Hippuric Acid Liver Function Test in Children, J. Pediat. 18: 371, 1941.
46. Wintrobe, M. M.: The Erythrocyte Sedimentation Test, Am. J. Clin. Path. 11: 562, 1941.

113 W. Chestnut Hill Avenue

SOLUBILITY AND pH DATA OF SOME OF THE COMMONLY USED SULFONAMIDES*

William G. Clark, Ph.D., Ernest A. Strakosch, M.D., M.S., and Nathan I. Levitan, M.S., Minneapolis, Minn.

While working in the field of the local application of sulfonamides to infections, we frequently needed solubility and pH data of the sulfonamides. A search of the literature has failed to reveal a source of collected data on these physical properties of the more commonly used sulfonamides. The data which exist are very scattered and frequently appear in papers which are abstracted in such a way as to disguise the fact that such data do appear in the papers. Moreover, some data are lacking in the literature we have been able to examine, including brochures and pamphlets of various commercial firms.

Hence it was thought that if these data were made available in collected form in a single table, it would prove extremely useful to workers in the fields of chemotherapy, particularly at the present time. For this reason, we have determined the solubilities of the commonly used sulfonamides and their sodium salts at room temperature and at body temperature in water and in human serum. In addition, we have determined the pH values of the saturated aqueous solutions, since this information is also lacking in many instances.

EXPERIMENTAL

A small tinted glass container containing excess sulfonamide in carbon dioxide-free distilled water or in serum, was shaken in a water bath thermostatically controlled to within 0.1° C. for twenty-four hours. The saturated solution was then filtered by aspiration through a washed and dried asbestos filter stick into a weighed weighing bottle. The entire apparatus was kept at the temperature at which the compound was dissolved (25° C. or 37° C.). The amount dissolved was then determined by the method of Bratton and associates,[1] using a photoelectric colorimeter. The pH values in carbon dioxide-free distilled water were determined at 25° C. with a Coleman pH meter (glass electrode/calomel electrode). The results are tabulated in Table I, along with values collected from the literature by other workers.

The large difference in solubility of sulfathiazole and sulfadiazine in water versus serum are striking, especially the latter. It has been pointed out by Roblin and others,[8] that blood levels exceed the solubility of sulfadiazine in water, while the blood levels obtained with the other sulfonamides are below the water solubility.

*From the Division of Physiology, Department of Zoology, and the Division of Dermatology and Syphilology, University of Minnesota, Minneapolis.

Supported by grants from the General Research Fund of the University of Minnesota, and the Abbott Laboratories, North Chicago, Ill. Assistance was furnished by the personnel of Work Projects Administration, O.P. 165-1-71-124, Subproject 383.

Received for publication, July 15, 1942.

TABLE I

SOLUBILITY AND pH DATA OF SOME OF THE COMMONLY USED SULFONAMIDES
(% = grams per 100 Gm. of the solvent)

Compound	25° C. Temperature					37° C. Temperature			
	Water (%)	Human Serum (%)	pH*	Refs.	Remarks	Water (%)	Human Serum (%)	Refs.	Remarks
Sulfanilamide	0.836	0.981	7.15	L†		1.460	1.970	L	
	0.750	-----	-----	7		1.500	-----	4	
Sulfathiazole	0.0502	0.234	5.90	L	At 26° C.	0.0960	0.330	L	
	0.0600	-----	-----	2		0.0942	-----	10	
						0.0940	-----	8	
						-----	0.184	4	At 36° C.
Sulfapyridine	0.0268	0.053	6.65	L		0.0486	0.0756	L	
	0.0280	-----	-----	7		0.0495	-----	5	
						0.0490	-----	7	
						0.0530	-----	9	
						-----	0.0610	4	At 36° C.
Sulfadiazine	0.0077	0.115	5.75	L		0.0127	0.161	L	
						-----	0.124	4	At 36° C.
						0.0123	-----	8	
						0.0120	0.107**	10	
						-----	0.247***	10	
Sodium sulfathiazole	45.0		9.65****	L	Coagulates serum	60.0		L	Coagulates serum
	45.0			3					
Sodium sulfapyridine	52.0		10.85	L	Coagulates serum	80.0		L	Coagulates serum
	54.0			6					
Sodium sulfadiazine	50.0		9.90	L	Coagulates serum	65.0		L	Coagulates serum

*All pH determinations were made in carbon dioxide-free water solutions.
†L refers to our determinations. The numbers refer to those of others.
**Horse serum.
***Defibrinated rabbit blood.
****The pH values for 0.01 molar aqueous solutions were found to be:

Sodium sulfathiazole	pH 9.20
Sodium sulfapyridine	pH 10.10
Sodium sulfadiazine	pH 8.75

We are at present determining similar data for the sulfonamides in other solvents which may be of more practical use in the topical treatment of infections.

REFERENCES

1. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. 128: 537, 1939.
2. Lott, W. A., and Bergeim, F. H.: (p-Aminobenzenesulfonamido)-Thiazole: A New Chemotherapeutic Agent, J. Am. Chem. Soc. 61: 3593, 1939.
3. Pharmacological Activity and Clinical Use of Sulfathiazole, Merck & Co., Inc., Rahway, N. J., April, 1942, page 1.
4. Hawking, F.: Local Concentration of Sulphonamide Compounds Inserted Into Wounds, Lancet 240: 786, 1941.
5. Hug, E.: Solubility of Sulfapyridine and Acetylsulfapyridine Under Various Conditions, Rev. Soc. argent. de biol. 16: 662, 1940.
6. Marshall, E. K., Jr., Bratton, A. C., and Litchfield, J. T., Jr.: The Toxicity and Absorption of 2-Sulfanilamidopyridine and Its Soluble Sodium Salt, Science 88: 597, 1938.
7. Clinical Use of Sulfanilamide Compounds in the Treatment of Gonococcal Infections, Merck & Co., Inc., Rahway, N. J., Nov., 1941, pp. 1-3.
8. Roblin, R. O., Jr., Williams, J. H., Winnek, P. S., and English, J. P.: Chemotherapy. II. Some Sulfanilamido Heterocycles, J. Am. Chem. Soc. 62: 2002, 1940.
9. Roblin, R. O., Jr., and Winnek, P. S.: Chemotherapy. I. Substituted Sulfanilamidopyridines, J. Am. Chem. Soc. 62: 1999, 1940.
10. Feinstone, W. H., Williams, R. D., Wolff, R. T., Huntington, E., and Crossley, M. L.: The Toxicity, Absorption and Chemotherapeutic Activity of 2-Sulfanilamidopyrimidine (Sulfadiazine), Bull. Johns Hopkins Hosp. 67: 427, 1940.

FAT METABOLISM IN ACNE VULGARIS*

Edward B. LeWinn, M.D., and I. Zugerman, M.D.
Philadelphia, Pa.

WHILE the relationship between fat metabolism and acne vulgaris has been suggested previously,[1] no studies of this problem have been made by methods which reflect the status of the fat metabolism in this skin disease. It has also been proposed that acne vulgaris is of endocrine origin, but up to the time of the present report the investigations have been disappointing, the results permitting no certain conclusions. Cholesterol is chemically related to the steroidal hormones, such as estrogens and androgens, the latter having been found to cause acne when given to women in very large doses.[2] On the other hand, cholesterol is intimately associated with the metabolism of fats. This dual relationship was utilized in a study of fat tolerance in acne vulgaris, herein reported, employing the blood cholesterol as an indicator of the blood lipid content.[3]

MATERIAL

In this investigation 20 patients, 12 females and 8 males, with acne vulgaris were studied. Their ages ranged from 11 to 29 years, the average being 19 years. The duration of their acne varied from one month to 11 years. The severity of the lesions varied from a few well-marked comedones and pustules on the forehead to extensive and active involvement of the forehead, face, shoulders, chest, and back. Most of the 12 female patients exhibited some menstrual disturbances, mainly irregularity or amenorrhea.

Thirteen patients without evidence of past or present acne were studied for comparison with the acne patients. These patients also served as controls in a study of fat metabolism in psoriasis.[3] There were 8 women and 5 men in the group, ranging in age from 20 to 65 years, the average being 41 years. The average age of the control group is considerably higher than that of the acne patients. However, advancing age causes only very slight changes in blood cholesterol levels.[4]

PROCEDURE

After a twelve-hour fast a blood specimen was taken. The patient then drank 500 c.c. of 20 per cent cream, representing 100 Gm. of fat. Blood specimens were then obtained at intervals of one and one-half, three, four and one-half, six, and seven and one-half hours, and the whole blood was examined for total cholesterol by the method of Bloor.[5] During the time of the test each patient was allowed to take an occasional sip of water. Most patients were ambulant but activity was closely restricted.

---

*From the Medical Service of Dr. Mitchell Bernstein and the Dermatological Service of Dr. A. Strauss, Jewish Hospital, Philadelphia.

Received for publication, August 7, 1942.

RESULTS

The fasting total cholesterol levels in 20 cases of acne vulgaris were between 125 mg. and 275 mg. per 100 c.c. of whole blood, the mean being 173 mg. In the 13 control cases the fasting cholesterol ranged from 123 to 250 mg. per 100 c.c.,

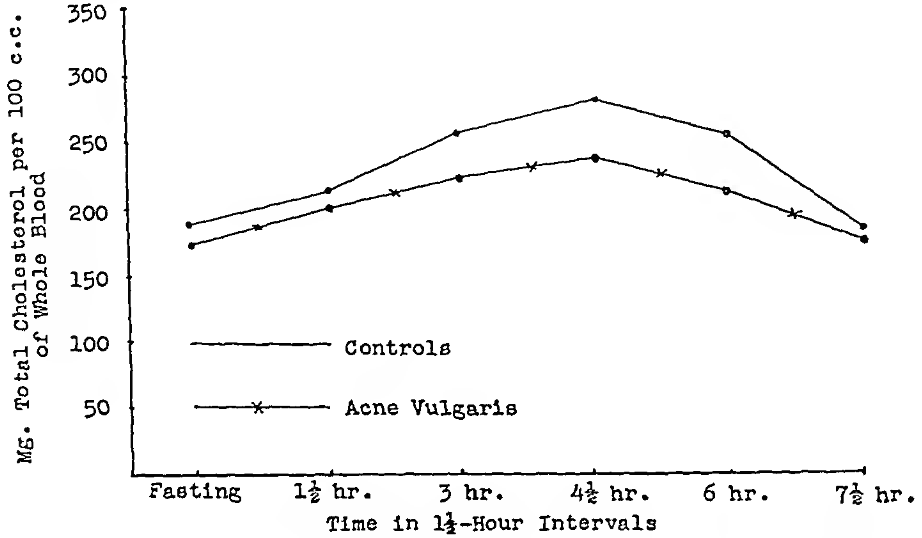


Fig. 1.—Composite blood cholesterol curves following the ingestion of fat.

the mean being 188 mg. In the acne patients the range of increases from fasting to the highest level of blood cholesterol was from 25 mg. to 208 mg. per 100 c.c. of whole blood, the mean being 83 mg. On the other hand, the control group showed increases above the fasting level of from 42 mg. to 198 mg., with a mean of 119 mg. (Table I).

TABLE I

PATIENTS WITH ACNE VULGARIS

CASE	FASTING	1½ HR.	3 HR.	4½ HR.	6 HR.	7½ HR.	MG. RISE
1	125	132	150	192	227	150	102
2	125	135	151	156	147	131	31
3	132	150	172	185	178	150	53
4	139	156	166	166	178	208	69
5	147	166	172	192	217	178	70
6	150	178	208	238	192	166	88
7	150	172	192	200	217	172	67
8	151	172	178	185	208	160	57
9	160	208	250	227	175	160	90
10	160	192	222	227	208	178	67
11	166	189	227	189	172	151	61
12	166	156	171	191	166	138	25
13	166	191	250	263	227	171	97
14	166	178	208	238	192	166	72
15	192	208	217	227	250	208	58
16	208	227	250	263	238	192	55
17	208	250	312	416	208	178	208
18	227	263	295	333	250	192	106
19	250	333	257	455	357	312	205
20	275	257	312	227	184	166	82

Data for control patients have been presented in an earlier publication.[3]

Examination of the curves of the means of the two groups does not reveal a significant difference between them in fat tolerance, all points of the composite curves of both groups being within accepted normal limits. The marked similarity between the acne and the nonacne patients is further emphasized by statistical examination of the figures. The application of the *t* test to the fasting levels of blood cholesterol gives a value of 0.3. The value of *t* for the peak levels of the two groups is 0.6. For groups of the size studied in this report *t* should have a value of 2.04 or greater to be considered significant. It is worthy of note that Strickler and Adams[6] were also unable to demonstrate any departure from normal in the fasting blood cholesterol levels of acne patients.

CONCLUSIONS

A study of the fat tolerance, as indicated by blood cholesterol changes following the ingestion of fat, of a group of 20 patients with acne vulgaris fails to reveal any difference from a group of patients without acne vulgaris.

Dr. E. D. Burdick, of the University of Pennsylvania, assisted in the statistical review of our results.

Dr. O. E. Helm, Jr., permitted us to study several of his patients in the Dermatological Service at Frankford Hospital.

REFERENCES

1. Sutton, R. L., and Sutton, R. L., Jr.: Diseases of the Skin, ed. 10, St. Louis, 1939, The C. V. Mosby Co.
2. Geist, S. H., Salmon, U. J., Gaines, J. H., and Walter, R. T.: The Biologic Effects of Androgen (Testosterone Propionate) in Women, J. A. M. A. 114: 1539, 1940.
3. LeWinn, E. B., and Zugerman, I.: Fat Tolerance Tests in Psoriasis, Am. J. M. Sc. 201: 703, 1941.
4. Parhon, C. J., and Parhon, M.: L'hypercholesterinemie de la vieillesse, Compt. rend. Soc. de biol. 88: 231, 1923.
5. Bloor, W. R.: The Determination of Small Amounts of Lipid in Blood Plasma, J. Biol. Chem. 77: 53, 1928.
6. Strickler, A., and Adams, P. D.: Cholesterol Metabolism in Certain Dermatoses, With Special Reference to Acne Vulgaris, Arch Dermat. & Syph. 26: 11, 1932.

LABORATORY METHODS

GENERAL

A MODIFICATION OF THE KAHN TEST FOR USE WITH HEMOLYZED BLOOD*

H. J. LAWLER, Sc.D., ST. LOUIS, MO.

IN LABORATORIES which perform diagnostic tests for syphilis, one difficulty is that presented by blood specimen that has undergone so much hemolysis that precipitation tests cannot be read. The physician is usually notified that the specimen is not suitable for diagnostic test, and he is asked to send another sample of blood. There is inconvenience to both physician and patient.

Some idea as to the frequency of such hemolyzed specimens may be gained from the following tabulation of the last two years' experience of the St. Louis City Bacteriological Laboratory, where Kahn tests are performed on blood specimens collected by messenger from various points throughout the city each evening and tested the day after the blood is drawn.

	1940	1941
Total number of Kahn tests run	55,518	74,918
Number hemolyzed	343	689
Percentage hemolyzed†	59	91

The sharp increase in the number of hemolyzed specimens in the latter year is probably related to the fact that many of the specimens submitted in connection with the selective service physical examinations were drawn by physicians who had previously had relatively little experience with the submission of specimens to the municipal laboratory. Those physicians who send them in frequently are apparently more aware of the precautions which aid in the prevention of hemolysis.

When the percentages of hemolyzed specimens received by the laboratory are graphically represented in relation to the months of the year, as in Fig. 1, it is apparent that the problem is accentuated during the summer months. This was particularly true during 1941, when approximately four such specimens were received by the laboratory each working day from May through September.

*From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis.

†Included as hemolyzed are those specimens containing enough hemoglobin to obscure completely the reading of the Kahn test.

Received for publication, April 24, 1942.

The best solution of the problem would be the prevention of hemolysis, and much of it can be prevented by reminding the physician of the simple precautionary measures:

a. Use of a dry syringe and a fairly large needle.
b. Permit the blood to flow into the syringe, with a minimum of negative pressure.
c. Remove the needle from the syringe before *gently* emptying the syringe.
d. If feasible, place the specimen in a refrigerator, especially if there may be delay in getting it to the laboratory.

If, however, hemolyzed specimens are sent in, then the laboratory might well make use of them if adequate modification of existing methods is made available to assure the reliability of such tests. Any satisfactory method should remove the hemoglobin, should give the same results that would have been obtained with clear serum from the same source, and should be adapted to the equipment and skills of the usual public health laboratory.

The particular method described herein is essentially a modification of the usual Kahn test for spinal fluid. The serum is diluted with a relatively large volume of ammonium sulfate solution to "salt" out the globulin; this is centrifuged to a minimum volume. The supernatant solution, containing most of the hemoglobin of the original specimen, is poured off, the globulin is redissolved, and a slightly modified Kahn test is performed on the globulin solution.

MATERIALS AND METHODS

Syphilitic sera for testing the efficiency of the proposed method were obtained from the St. Louis City Bacteriological Laboratory. Only weakly positive and negative sera were used, for it was found that strongly positive sera gave uniformly positive tests even with less adequate modifications of the method and, therefore, could not be used in comparative evaluations. "Hemolyzed specimens" were prepared from these sera by the addition of "hemoglobin solution" prepared in the following manner: Packed red blood cells from nonsyphilitic blood were alternately frozen and thawed until hemolyzed. After removal of stroma by centrifugation, the resulting serum was adjusted to contain 20 Gm. of hemoglobin per 100 ml., as determined by the cyanmethemoglobin method in the Evelyn colorimeter (Evelyn and Malloy, 1938), and 0.2 ml. of this "hemoglobulin solution" was added to 0.8 ml. of serum of known syphilitic titer to produce 1 ml. of a hemolyzed specimen. These proportions were determined by estimation that hemolysis sufficient to obscure completely the Kahn precipitate might add 20 per cent to the volume of the serum.

The Kahn tests were performed in the manner prescribed by Kahn (1928). They were read immediately after the addition of saline, and again after fifteen minutes; the amounts of precipitate in the three tubes are expressed in the tables in this paper in terms of plus signs. The results of the second reading are recorded above the results of the first, if there was any change.

PREPARATION OF GLOBULIN SOLUTION

1. The specimen is subjected to centrifugation to remove cells, clot fragments, etc., as in the usual preparation of serum (fifteen minutes at 2,000 r.p.m.).

2. One milliliter of the supernatant fluid is pipetted off into a small round-bottomed test tube (the 100 by 12 mm. size is convenient), and after addition of 5 ml. of ammonium sulfate solution* the mixture is incubated at 56° C. for fifteen minutes.
3. The precipitate which is formed is separated by centrifuging at 2,000 r.p.m. for twenty minutes. Supernatant fluid is poured off, and the tube containing the precipitate is inverted over absorbent paper to remove as much of the salt solution as is convenient.
4. The sediment in the tube is dissolved by the addition of 1.3 ml. of distilled water, while stirring with a wooden applicator stick, and after centrifuging for five minutes at 1,500 r.p.m., the supernatant is transferred into another tube by means of a capillary pipette.
5. Three tube Kahn test is now set up, using 0.15 ml. of this globulin solution (instead of serum) and the usual amounts of antigen (Kahn, 1928) in each tube. The mixture is shaken at the rate of 275 times per minute for *four* minutes instead of three.

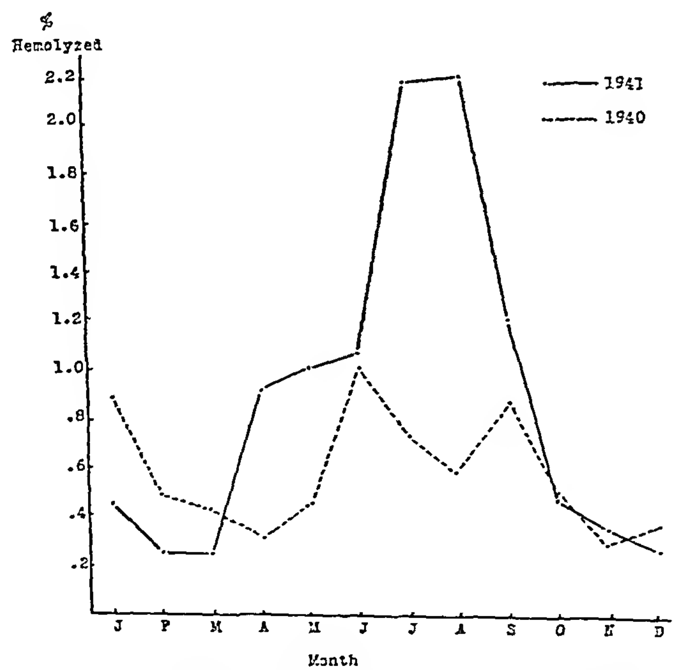


Fig. 1.—The occurrence of hemolyzed specimens among those received during 1940 and 1941 by the St. Louis Municipal Bacteriological Laboratory.

The reliability of this procedure was tested by using it with (a) "hemolyzed specimens" prepared as outlined previously, and (b) similar specimens were prepared by the addition of negative serum to the syphilitic sera in place of "hemoglobin solution." In Table I are listed the results of such tests, along with the results of standard Kahn tests run simultaneously on twenty-four different sera.

In general, these comparative tests showed good agreement between the standard Kahn and the modified test with globulin solution; the greatest varia-

*The solution is prepared by adding 48 Gm. of "analytical reagent" grade ammonium sulfate to 100 ml. of distilled water. In adding this to the serum, blowing it in is an easy method of mixing.

tion between the two in the case of any one serum was ++. The reagin-antigen precipitates were slightly smaller when formed from the globulin solution than when formed from whole serum, although the recommended extra minute's shaking given the antigen-globulin mixture tends to make this difference less pronounced.

An additional observation, possibly bearing on the evaluation of the modified test, was made in the case of globulin prepared from known negative sera: doubtful reactions (±) were frequent, and one-plus precipitates were formed in the middle tube in several cases. The particles of these precipitates were smaller and more evenly distributed than the usual Kahn precipitates.

TABLE I

RESULTS OF A MODIFIED KAHN TEST ON SOLUTIONS OF GLOBULIN COMPARED WITH THE RESULTS OF THE STANDARD KAHN TEST ON THE UNALTERED SERA

SERUM NO.	GLOBULIN PRECIPITATED FROM (A) 0.8 ML. POSITIVE SERUM + 0.2 ML. "HEMOGLOBIN SOLUTION"			GLOBULIN PRECIPITATED FROM (B) 0.8 ML. POSITIVE SERUM + 0.2 ML. NEGATIVE SERUM			CONTROL KAHN TEST ON ORIGINAL SERA		
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	+++ +	++++	++++	++	++++	++++	++++	++++	++++
2	++ +	++++	++++	−	++++	++++	±	++++ +++	++++
3	++ −	++++ +++	++++	±	++++	++++	−	++++	++++
4	±	++++	++++	±	++++	++++	−	++	++++
5	−	±	±	−	+	−	−	−	−
6	−	+ ++	−	−	±	±	−	− ++	−
7	−	+++	++++	−	+++	++++	−	++++ +	++++
8	−	+++	++++	−	+++ ++	++++	−	+++	++++
9	−	++++	++++	− ++	+++	++++	−	++++	++++
10	+++	++++	++++	+++ +++	++++	++++	++++	++++	++++
11	++++	++++	++++	++++	++++	++++	++++	++++	++++
12	++++	++++	++++	++++	++++	++++	++++	++++	++++
13	−	−	±	−	+	−	−	−	−
14	−	+	−	−	−	−	−	−	−
15	−	−	±	−	±	±	−	−	−
16	++	++++	++++				+++	++++	++++
17	++++	++++	++++				++++	++++	++++
18	++	++++	++++				−	++++	++++
19	+	++	++				−	±	+++
20	±	+	−				−	−	−
21	+	++++	++++				+	++	++++
22	++	++++	++++				+	++++	++++
23	−	±	−				−	−	−
24	−	−	−				−	−	−

FACTORS AFFECTING THE MODIFIED TEST

1. The amount of fluid used in dissolving the globulin.

Using four "hemolyzed specimens," prepared as outlined previously, parallel modified Kahn tests were run on solutions of globulin made by the addition of 0.8, 1.0, 1.2, and 1.4 ml. of 0.9 per cent saline solution to the precipitates. The results of these tests and of the simultaneously run standard Kahn tests on the original sera (Table II) showed the closest correspondence in the case

TABLE II

RESULTS OF THE MODIFIED KAHN TEST ON GLOBULIN SOLUTIONS MADE UP WITH VARIOUS AMOUNTS OF SALINE

SERUM NO.	AMOUNT OF SALINE ADDED TO GLOBULIN												CONTROL KAHN TEST ON ORIGINAL SERA		
	0.8 ML.			1.0 ML.			1.2 ML.			1.4 ML.					
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	++	++++	–	+++	++++	++++	+ ++	+++ ++++	++++	+	++++	++++	+ ++	++++	++++
2	++++	+++	–	++++	++++	+++	++++	++++	++++	+++	++++	++++	++++	++++	++++
3	+++	++++	–	+ ++	+++ ++++	++++	∓	+ +++	++++	–	+++	++++	+∓	++ ++++	++++
4	–	++++	++	∓	+++	+++	–	++ +++	++++	–	–	++++	–	+ ++	++++
5	+	++	++	–	+	++	–	±	±	–	±	–	–	–	–

TABLE III

RESULTS OF THE MODIFIED KAHN TEST ON SOLUTIONS OF GLOBULIN PRECIPITATED FROM SERA CONTAINING VARIOUS AMOUNTS OF HEMOGLOBIN

SERUM NO.	CONTENT OF SPECIMEN												CONTROL KAHN TEST ON ORIGINAL SERA		
	0.9 ML. SERUM + 0.1 ML. HB. SOLUTION			0.9 ML. SERUM + 0.2 ML. HB. SOLUTION			0.8 ML. SERUM + 0.3 ML. HB. SOLUTION			0.7 ML. SERUM + 0.4 ML. HB. SOLUTION					
	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
1	–	++ ++++	++++ 2	–	++ +++	++++ 2	–	++	++++ 2	–	+++ ++	++++ 2	–	++ ++++	++++ 2
2	–	++	++++ 2	–	+ ++	++++ 2	–	+++	++++ 2	–	+++ ++	+++ ++ 2	–	+ ++∓	++++ 2
3	–	++++	++++ 3	–	++++	++++ 3	–	+++	++++ 2	–	+++	+++ ++ 2	–	++++	++++ 3
4	–	–	– –	–	–	– –	–	±	± –	–	–	+ –	–		– –

of the solution made with 1.2 ml. of saline. More than that amount (1.4 ml.) seemed to make the test less sensitive, while less (1.0 ml.) made it more sensitive, negative serum number 5 showing a doubtful positive. Still less produced bizarre reactions (see Table II).

2. The effect of increased amounts of hemoglobin.

Four sets of "hemolyzed specimens" were prepared, containing different amounts of "hemoglobin solution," as outlined in Table III. Modified Kahn tests on globulin precipitated from these gave results very similar to those obtained with standard Kahn tests on the sera; the added hemoglobin apparently interfered little, except in the case of the specimens prepared with 0.4 ml. of "hemoglobin solution." In these the second reading showed increases in the amounts of precipitate. In possible explanation of this it was noticed that the volume of the precipitated, centrifuged globulin in these tubes was about double that precipitated from those having only 0.1 ml. of "hemoglobin solution." The rest of the results, with specimens containing smaller amounts of "hemoglobin solution," so closely paralleled the control results that it seems likely the effect of hemoglobin on the globulin precipitation is probably small, within a rather wide range of concentration.

3. The ammonium sulfate solution.

It is recommended that "analytical reagent" grade of this salt be used, so that solutions used for globulin precipitation would be approximately the same regardless of the source of the chemical. In order to insure that the solution would be half saturated even at summer temperatures, it is made up with 48 Gm. added to each 100 ml. of distilled water. Five milliliters of this solution, plus 1 ml. of the serum from the hemolyzed specimen results in a solution that would be half saturated with ammonium sulfate at any temperature below 36° C.

SUMMARY

The problem of hemolysis in blood submitted to the St. Louis Municipal Bacteriological Laboratory is discussed. A method for performing a modified Kahn test on such "hemolyzed specimens" is described, and the results of comparative tests with this method and the standard Kahn test are given.

The modified Kahn test described herein for use with hemolyzed blood has been found to give approximately the same results as the standard Kahn test in comparative tests with a limited number of specimens. It is, therefore, being recorded with the view of suggesting comparative trial in laboratories which find hemolysis a definite problem.

I wish to express my gratitude to Dr. J. J. Bronfenbrenner and Mr. Nathan Nagle for advice and assistance in connection with the work.

REFERENCES

Evelyn, K. A., and Malloy, H. T.: Microdetermination of Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin in a Single Sample of Blood, J. Biol Chem. 126: 655, 1938.

Kahn, R. L.: The Kahn Test—A Practical Guide, Baltimore, 1928, Williams & Wilkins Co.

A SIMPLE AUTOMATIC DISTILLING UNIT*

Bradford N. Craver, Ph.D., and Howard L. Williamson, B.Ed*
Detroit, Mich.

The problem of redistilling two or three gallons of water every day can be troublesome when the largest flask available for a still has only the capacity of a liter. The use of a much larger flask is not satisfactory when only small burners are available for heating it. Accordingly, we devised the apparatus herein sketched to solve the problem. It permits continuous distillation throughout the day without, in our experience, requiring the slightest attention. A hasty survey of the literature plus consultation with our associates has failed to reveal a similar arrangement. Albeit, since the principles involved are so simple, they have undoubtedly been combined before to the same end. Hence, we make claim to nothing more than independent discovery. However, with the realization that other laboratories with limited apparatus may face a similar problem, we considered it worth while to make available a detailed description of the unit.

Essentially the siphon principle is combined with that of the Mariotte bottle. The bottom of the tube, *B*, in the Mariotte bottle, *A*, is adjusted so as to lie in the same horizontal plane as the surface of the water in the flask, *C*, when the flask has been filled to a level convenient for distillation. In practice the fluid usually rises somewhat higher in the flask *C* than the level calculated because the air in the Mariotte bottle *A* becomes progressively hotter as the distillation proceeds, due to the small amount of steam which bubbles back through the tube *D*. Accordingly, we usually adjust the Mariotte bottle, so that the end of the tube, *B*, is in the same horizontal plane as the surface of the liquid in the flask *C*, when the flask is about two-thirds full. The end of the siphon tube, *D*, is bent slightly upward and outward so that the orifice is near the wall of the flask. This tends to minimize the amount of steam which always bubbles back up through the tube, *D*, into the Mariotte bottle. Although this steam heats the water in the Mariotte bottles as the distillation progresses, in our experience this has neither interfered with the smooth operation of the unit nor cracked the nonpyrex Mariotte bottle. The lower opening of the tube, *D*, must, of course, be below the lower end of the tube, *B*, or the water will fail to siphon into flask *C*, as the distillation boils off the fluid in flask *C*. *H* is merely a plug of glass wool to prevent the entrainment of impure water droplets in the steam which passes over into the condenser, *E*. Any flask may serve as a receiver, *F*. *G* is a cotton plug to guard the opening of the receiver, *F*, so that no dust can fall into the receiver. The area of the condenser surface, if too small, will, of course, limit the rate at which one can distill since some of the steam, when too small a

*From the Department of Pharmacology, Wayne University College of Medicine, Detroit.
Received for publication, April 22, 1942.

of the solution made with 1.2 ml. of saline. More than that amount (1.4 ml.) seemed to make the test less sensitive, while less (1.0 ml.) made it more sensitive, negative serum number 5 showing a doubtful positive. Still less produced bizarre reactions (see Table II).

2. The effect of increased amounts of hemoglobin.

Four sets of "hemolyzed specimens" were prepared, containing different amounts of "hemoglobin solution," as outlined in Table III. Modified Kahn tests on globulin precipitated from these gave results very similar to those obtained with standard Kahn tests on the sera; the added hemoglobin apparently interfered little, except in the case of the specimens prepared with 0.4 ml. of "hemoglobin solution." In these the second reading showed increases in the amounts of precipitate. In possible explanation of this it was noticed that the volume of the precipitated, centrifuged globulin in these tubes was about double that precipitated from those having only 0.1 ml. of "hemoglobin solution." The rest of the results, with specimens containing smaller amounts of "hemoglobin solution," so closely paralleled the control results that it seems likely the effect of hemoglobin on the globulin precipitation is probably small, within a rather wide range of concentration.

3. The ammonium sulfate solution.

It is recommended that "analytical reagent" grade of this salt be used, so that solutions used for globulin precipitation would be approximately the same regardless of the source of the chemical. In order to insure that the solution would be half saturated even at summer temperatures, it is made up with 48 Gm. added to each 100 ml. of distilled water. Five milliliters of this solution, plus 1 ml. of the serum from the hemolyzed specimen results in a solution that would be half saturated with ammonium sulfate at any temperature below 36° C.

SUMMARY

The problem of hemolysis in blood submitted to the St. Louis Municipal Bacteriological Laboratory is discussed. A method for performing a modified Kahn test on such "hemolyzed specimens" is described, and the results of comparative tests with this method and the standard Kahn test are given.

The modified Kahn test described herein for use with hemolyzed blood has been found to give approximately the same results as the standard Kahn test in comparative tests with a limited number of specimens. It is, therefore, being recorded with the view of suggesting comparative trial in laboratories which find hemolysis a definite problem.

I wish to express my gratitude to Dr. J. J. Bronfenbrenner and Mr. Nathan Nagle for advice and assistance in connection with the work.

REFERENCES

Evelyn, K. A., and Malloy, H. T.: Microdetermination of Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin in a Single Sample of Blood, J. Biol Chem. 126: 655, 1938.

Kahn, R. L.: The Kahn Test—A Practical Guide, Baltimore, 1928, Williams & Wilkins Co.

A SIMPLE AUTOMATIC DISTILLING UNIT*

Bradford N. Craver, Ph.D., and Howard L. Williamson, B.Ed*
Detroit, Mich.

THE problem of redistilling two or three gallons of water every day can be troublesome when the largest flask available for a still has only the capacity of a liter. The use of a much larger flask is not satisfactory when only small burners are available for heating it. Accordingly, we devised the apparatus herein sketched to solve the problem. It permits continuous distillation throughout the day without, in our experience, requiring the slightest attention. A hasty survey of the literature plus consultation with our associates has failed to reveal a similar arrangement. Albeit, since the principles involved are so simple, they have undoubtedly been combined before to the same end. Hence, we make claim to nothing more than independent discovery. However, with the realization that other laboratories with limited apparatus may face a similar problem, we considered it worth while to make available a detailed description of the unit.

Essentially the siphon principle is combined with that of the Mariotte bottle. The bottom of the tube, *B*, in the Mariotte bottle, *A*, is adjusted so as to lie in the same horizontal plane as the surface of the water in the flask, *C*, when the flask has been filled to a level convenient for distillation. In practice the fluid usually rises somewhat higher in the flask *C* than the level calculated because the air in the Mariotte bottle *A* becomes progressively hotter as the distillation proceeds, due to the small amount of steam which bubbles back through the tube *D*. Accordingly, we usually adjust the Mariotte bottle, so that the end of the tube, *B*, is in the same horizontal plane as the surface of the liquid in the flask *C*, when the flask is about two-thirds full. The end of the siphon tube, *D*, is bent slightly upward and outward so that the orifice is near the wall of the flask. This tends to minimize the amount of steam which always bubbles back up through the tube, *D*, into the Mariotte bottle. Although this steam heats the water in the Mariotte bottles as the distillation progresses, in our experience this has neither interfered with the smooth operation of the unit nor cracked the nonpyrex Mariotte bottle. The lower opening of the tube, *D*, must, of course, be below the lower end of the tube, *B*, or the water will fail to siphon into flask *C*, as the distillation boils off the fluid in flask *C*. *H* is merely a plug of glass wool to prevent the entrainment of impure water droplets in the steam which passes over into the condenser, *E*. Any flask may serve as a receiver, *F*. *G* is a cotton plug to guard the opening of the receiver, *F*, so that no dust can fall into the receiver. The area of the condenser surface, if too small, will, of course, limit the rate at which one can distill since some of the steam, when too small a

*From the Department of Pharmacology, Wayne University College of Medicine, Detroit. Received for publication, April 22, 1942.

condenser is employed, will pass over into the receiver uncondensed. In actual operation the distillation is started with water in flask *C*, at the usual level, about two-thirds full. As the water in *C* boils down to a critical level about one inch above the bottom of the flask, water begins to siphon over into the still, *C*, from the Mariotte bottle, *A*. This cool water stops the boiling until flask *C* is again about two-thirds full, at which point siphoning ceases. With each succeeding filling the water will rise to a slightly higher level in flask *C*, because the air in the

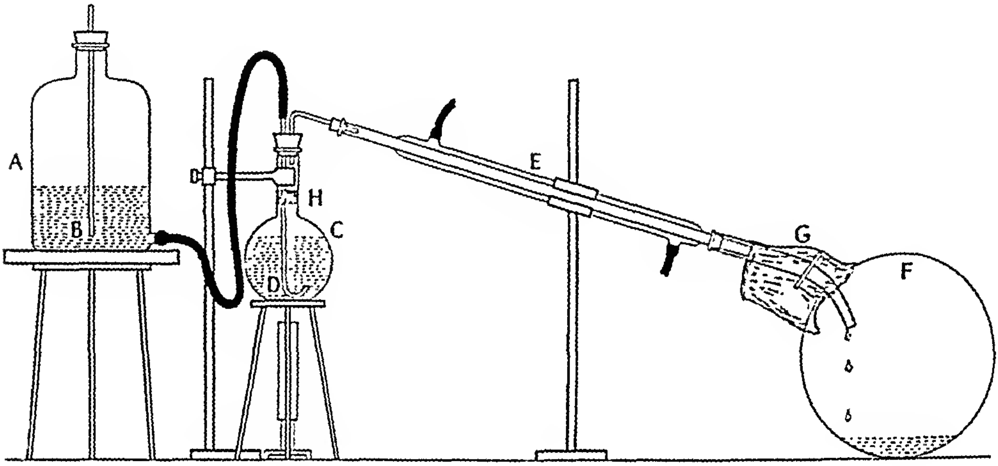


Fig. 1.

Mariotte bottle, as already noted, grows hotter as the distillation continues. All connections must be airtight and this precaution applies especially to the stopper in the top of the Mariotte bottle. With the precautions mentioned the distillation can be started early in the day and will smoothly proceed unattended as long as there is water in the Mariotte bottle. With our 10-liter Mariotte bottle it runs all day.

We offer this device in the hope it may prove of equal convenience to other laboratories faced with a similar problem. This arrangement is now being utilized by associates in other departments.

A METHOD FOR RAPID, REPEATED, APPROXIMATE DETERMINATIONS OF THE TRANSVERSE DIAMETER OF THE HEART

SAMUEL WALDMAN, M.D., BROOKLYN, N. Y.

THE routine determination of the transverse diameter of the heart is usually accomplished by means of teleoradiography, orthodiagraphy, and fluoroscopy.[1]

The teleoradiogram is the x-ray film of the heart, conventionally taken at a distance of six feet. This technique involves the use of x-ray equipment and x-ray film. In orthodiagraphy the examiner draws the outline of the heart in the visualized plane. This method requires much time and skill. With the fluoroscope, a direct examination is made, and contours can be studied in active motion.

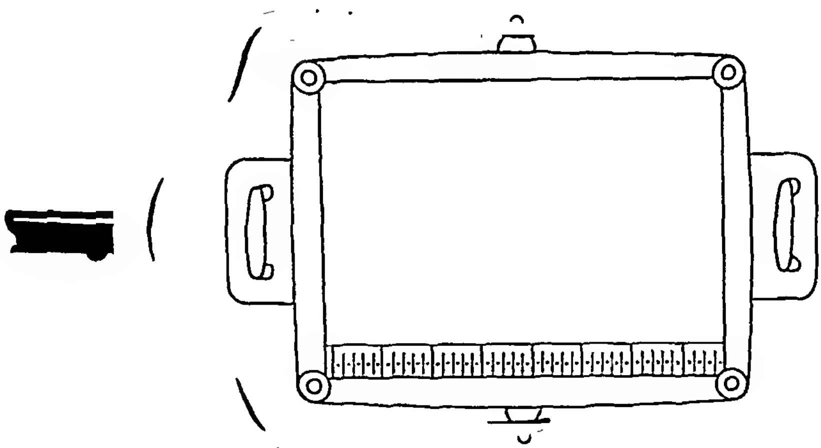


Fig. 1.

In order to determine rapidly and approximately the transverse diameter of the heart, I have devised a simple attachment to the fluoroscopic screen which, in a small degree, obviates the use of film and the orthodiagram, but which is not intended to supplant them.

For the conventional 12 by 16 inch fluoroscopic screen, a strip of x-ray film, ¾ by 16 inches, is cleared of emulsion by soaking in hot water. When it is thoroughly dry, lines are drawn on it with India ink as per diagram (Fig. 1). The width of the strip in the diagram is exaggerated for illustrative purposes. The ink is radiopaque and is easily seen during fluoroscopy of the chest. The complete lines indicate 5 cm., the incomplete lines 1 cm., and the dots 0.5 cm. Thus the accuracy is immediately limited to within 0.5 cm. The first line is drawn just within the edge of the screen. When ready, the strip is inserted into the base of the screen.

Received for publication, May 5, 1942.

By raising and lowering the screen, the widest diameter of the heart can be projected to this base line, and the measurement noted at once, giving the transverse diameter of the heart.

The patient must always stand touching his back to the fluoroscope, while the screen rests against the chest wall, and the head faces directly forward. The reading is taken at the end of inspiration. Under these conditions the measurements will be consistent and accurate. The figure obtained remains constant for the same patient under the same conditions. Repeated observations have borne this out. Little practice is required for rapid acquisition of the technique.

TABLE I

NAME	AGE	SEX	WEIGHT	HEIGHT (INCHES)	TRANSVERSE DIAMETER OF THE HEART		
					PREDICTED	OBTAINED	DEVIATION
Normal Hearts							
A. G.	27	M	145	67	125	130	5
H. W.	32	F	128	63	121	130	9
L. W.	24	F	130	60½	125	125	0
M. T.	33	F	157	62	135	130	5
A. S.	11	F	130	61	124	130	6
A. D.	29	F	108	62	112	120	8
L. W.	46	M	155	63	134	135	1
S. R.	26	F	136	63	125	130	5
S. D.	34	M	164	64	136	135	1
C. L.	24	F	101	59	110	110	0
J. G.	21	M	140	68	122	120	2
A. G.	19	M	171	69	134	135	1
E. W.	27	F	140	64	126	130	4
G. W.	24	F	169	68½	133	140	7
F. G.	43	F	137	62½	126	120	6
Hypertensive Hearts							
A. P.	49	F	181	62	146	165	19
F. K.	50	F	125	60	123	170	47
B. W.	61	M	145	64	128	160	32
M. R.	54	M	158	67	130	190	60
C. C.	51	M	146	64	128	142	14

This method of measurement has its greatest advantages when repeated studies of cardiac size are desired, especially at no material expense. Where changes in cardiac size occur, definite alteration in diameter can be determined at once. In the reversibly enlarged hearts of beriberi, myxedema, arteriovenous fistula, and cardiac dilatation, repeated measurements become invaluable and are easily determined by this method. Progressive enlargement or retrogression in the size of the heart can be observed easily, and recorded. Studies by Garland and McKenney[2] on the reversibly enlarged heart of thiamin deficiency revealed changes in the transverse diameter of the heart, as measured on 6 foot films, averaging a 2.7 cm. decrease in diameter in an average of fifteen days. The extremes varied from 1.0 to 4.5 cm. decrease in diameter of the heart, following the administration of thiamin. Such changes can be easily determined by the method herein described, without the use of film. Numerical records thus obviate somewhat repeated orthodiagrams and teleoradiograms.

Because of the closeness of the chest to the tube, the rays which strike the thoracic cage borders are too divergent for even approximate determinations of thoracic diameters which might conform to any tables. For the same reason

the larger the heart the more does its measure deviate from predicted figures. In this way, changes in size are more exaggerated than is actual, and therefore, more easily determined. The most accurate results are obtained in the normal heart size.

Ungerleider and Clark[3] in 1939 prepared tables of the transverse diameters of the heart, with which these measurements of the normal heart conform. The greatest deviation in the clinically normal heart was 9 mm. The least was 0 mm. Where clinical heart disease with enlargement existed, the figures roamed far from the predicted table.

In Table I are presented 15 patients with normal hearts, and five patients with hypertrophied hearts associated with hypertension. The comparative results by this method are easily seen.

SUMMARY

A simple method of immediate approximate measurement of the transverse diameter of the heart is described, obviating to a certain extent the use of orthodiagrams and teleoroentgenograms. Repeated studies in the same patient give excellent comparative results, and changes in the size of the heart are easily observed and recorded. Film is conserved, and time and expense are reduced to a minimum.

REFERENCES

1. Nomenclature and Criteria for Diagnosis of Diseases of the Heart, Criteria Committee of the New York Heart Association, New York, 1940, page 77.
2. Garland, L. H., and McKenney, A. C.: The Roentgen Diagnosis of Vitamin Deficiency Cardiac Conditions, Radiology 38: 4, 1942.
3. Ungerleider, H. E., and Clark, C. P.: A Study of the Transverse Diameter of the Heart Silhouette With Prediction Table Based on the Teleoroentgenogram, Am. Heart J. 17: 92, 1939.

1661 Prospect Place

APPARATUS AND TECHNIQUE FOR MEASUREMENT OF VIBRATORY THRESHOLD AND OF VIBRATORY "ADAPTATION" CURVE*

CHARLES D. ARING, M.D., CINCINNATI, OHIO, AND W. O. FROHRING, B.S., CHAGRIN FALLS, OHIO

IN THE clinical study of neurologic disorders attempts have been made to determine the appreciation of vibration. Little is known concerning the physiologic factors underlying this phenomenon other than the fact that the integrity of certain neural pathways is essential.

In order to elucidate the influence of various physiologic factors, such as circulation, respiration, emotion, and such physical factors as temperature and pressure on the appreciation of vibration, it was necessary to consider quantitative procedures. Numerous attempts at quantitative measurement had been made with but little headway until 1929, when an instrument was constructed for Tilney by Henney.[1] Subsequently, Laidlaw and Hamilton[2] improved this instrument (pallesthesiometer) and employed it to study the vibratory thresholds of various body surfaces. In spite of improvements, however, certain shortcomings still existed in the apparatus. It was our aim to devise an instrument which would retain the simplicity of the one constructed by Laidlaw and Hamilton, and at the same time eliminate certain variables which seemed inherent in their apparatus.

DESCRIPTION OF APPARATUS

The apparatus which was devised for these studies is illustrated in Fig. 1, the wiring diagram in Fig. 2. A Raytheon automatic voltage regulator was employed to reduce the voltage variation to plus or minus 0.5 per cent. The vibrating applicator has a standard resistance of 90 ohms and differs from the instruments employed by other investigators in that it vibrates horizontally, rather than vertically, to the surface of the area. Since an increase of pressure would tend to dampen the amplitude of vibrations applied vertically to the skin area, horizontal vibration was chosen, for it is less likely to be affected by pressure variation.

Results of preliminary tests on several subjects indicated that a maximum voltage of 50 would cover the most practical range with a 5,000 ohm potentiometer if the latter were "even" wound rather than "taper" wound. A dial divided into 100 divisions gives a more nearly logarithmic function of the amplitude than does a dial calibrated in arithmetic units. The arbitrary use of the dial reading for recording determinations seems to be entirely practical for comparing results. Because of possibility of error in calibration

*From the Neurological Service, Cincinnati General Hospital, the University of Cincinnati College of Medicine, and the Research Laboratories of the S. M. A. Corporation.

Received for publication, May 15, 1942.

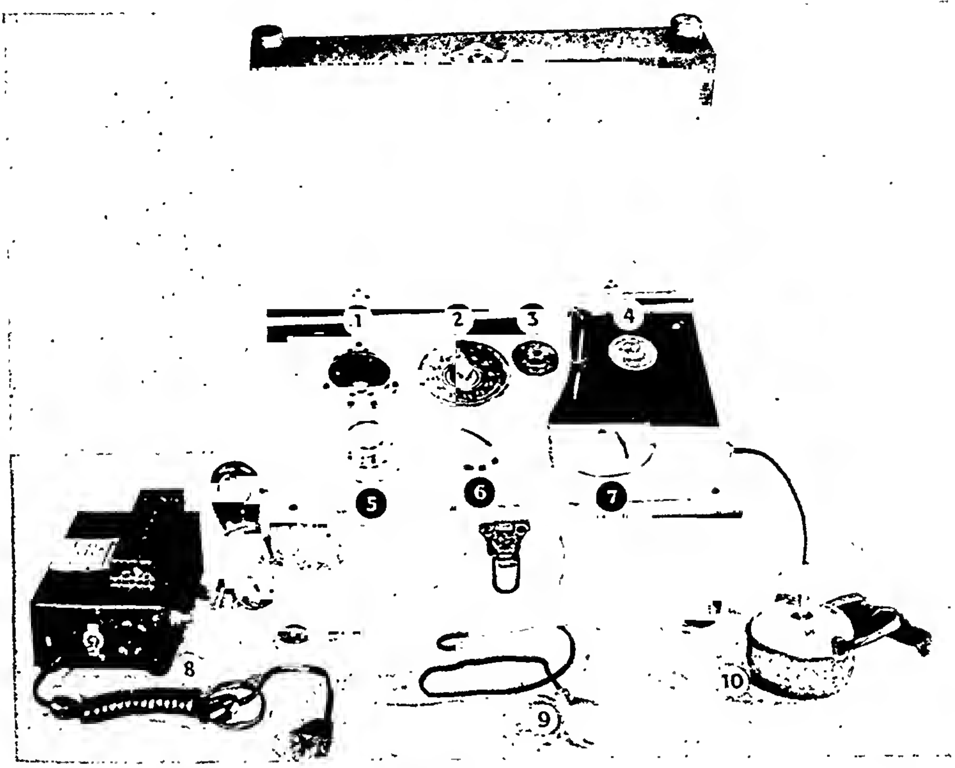


Fig. 1.—A portable pallesthesiometer.

1, Voltmeter.
2, Interval timer.
3, Switch for full amplitude of the vibrator.
4, Main switch.
5, and *6*, Voltage control knob.
7, Dial for recording amplitude of vibrator.
8, Electronic converter for use with direct current.
9, Push-button signal (operated by patient who indicates when vibration is felt).
10, Vibrator.

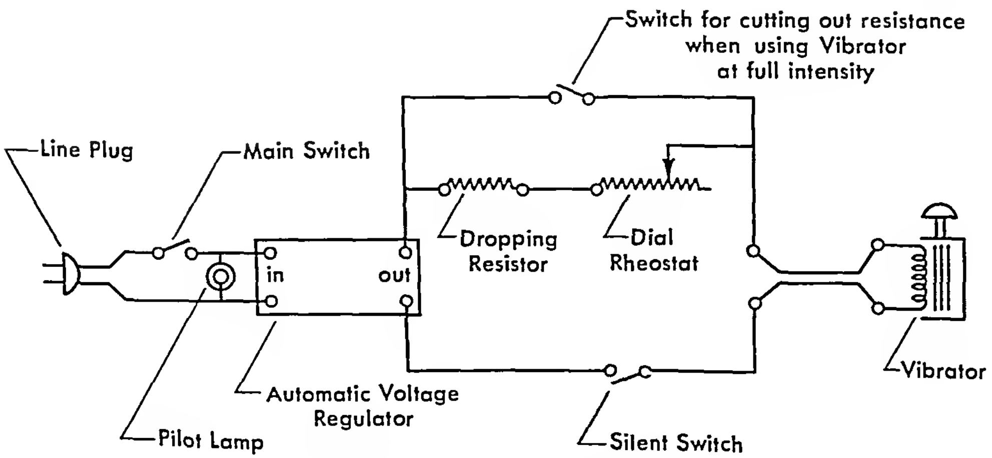


Fig. 2.—Wiring diagram of the pallesthesiometer.

methods, no attempt was made to calibrate the dial readings in terms of "units of energy" or "amplitude." In the preliminary studies, when the pallesthesiometer had an input of 110, the dial reading at zero (with the voltage across the vibrator) was 4½ volts; at a maximum dial reading of 93, 49 volts. In case the automatic voltage regulator is not used, a variable resistance must replace the dropping resistor in the vibrator circuit and a voltmeter must be placed across the leads to the vibrator. When only direct current is available, an electronic 60-cycle convertor may be inserted ahead of the instrument.

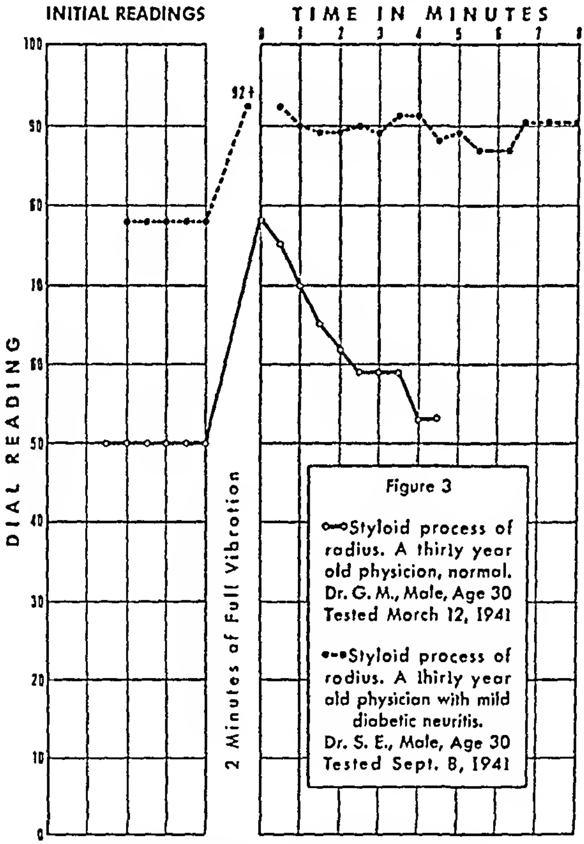


Fig. 3.—Vibratory "adaptation" curves.

METHOD

A modicum of attention is required on the part of the subject. After the procedure is explained, the subject may recline in the dorsal or ventral position. The vibrator is then applied to an area remote from that to be tested, so that the stimulus might be recognized before the test is begun. The applicator is held at right angles to the point tested, the weight of the entire applicator at that position representing roughly the pressure exerted on the skin. The initial threshold is then determined. With the dial set at zero and the vibrator applied to the test area, the dial is turned up slowly until the stimulus is perceptible. This process is repeated several times, allowing rest periods of thirty seconds to one minute between stimulations. It would be

logical to assume that threshold readings could be verified by starting with a stimulus heavier than the threshold, turning the dial toward zero, and recording the point at which the subject no longer perceives vibration. However, such a procedure results in an "adaptation" to the stimulus, particularly if the latter is heavy and is applied for more than an instant. Hence, it cannot be used to determine the threshold value.

The rate at which vibratory thresholds are approached has no effect on the final reading; that is to say, a variation in the speed at which readings are taken will not influence the subject's response. If he is reporting inaccurately, widely divergent readings are observed, indicating that the subject made his report when he thought the stimulus should have been evident rather than when it was actually felt.

The phenomenon of "adaptation" proved to be useful as a more delicate evaluation of vibratory appreciation than that educed by threshold determination alone. If, having determined the threshold of a surface over a given period of time, a heavy vibration was applied, the vibratory threshold was raised. When the "adapting" vibration was discontinued, it was found that the threshold returned to the original level in a period of a few minutes.

RESULTS

Vibratory thresholds were determined in normal persons. It was observed that readings in comparable test areas may vary widely from person to person. It is well known that the threshold varies from area to area on the body surface. Thus, Laidlaw and Hamilton[2] found that the vibratory threshold was lowest in the finger tips and highest at the sacrum.

Somewhat more constant was the adaptation to an intense stimulation. In the majority of subjects, return to the control threshold after a period of intense vibratory stimulation was observed in several minutes (Fig. 3). A much longer interval of return was observed in patients suffering from a variety of neurologic diseases (degeneration of the posterior columns of the spinal cord, subacute combined degeneration of the spinal cord, and peripheral neuritis) (Fig. 3). The influence of various physiologic and physical factors on vibratory appreciation in normal and abnormal states will be described in subsequent reports.

SUMMARY

An instrument for the quantitative measurement of the threshold of vibratory appreciation is described.

REFERENCES

1. Tilney, F.: Comparative Sensory Analysis of Helen Keller and Laura Bridgman; Its Bearing on Further Development of Human Brain, Arch. Neurol. & Psychiat. 21: 1237, 1929.
2. Laidlaw, R. W., and Hamilton, M. A.: Thresholds of Vibratory Sensibility as Determined by the Pallesthesiometer, Bull. Neurol. Inst. New York 6: 494, 1937.

A SIMPLE KIDNEY PLETHYSMOGRAPH*

FREDERICK E. EMERY, PH.D., BUFFALO, N. Y.

THE kidney can easily be approached, and by means of a little dissection can be freed from the abdominal wall and placed in a plethysmograph. Usually some difficulty is experienced in obtaining records of kidney volume because the plethysmographs adapted for the kidney have some disadvantages. *First*, they are difficult to make airtight; *second*, the vena cava or kidney vessels are often compressed, resulting in passive congestion; *third*, the instrument is frequently too large to be inclosed in the abdomen and, therefore, the temperature of the kidney may be below normal; and *fourth*, the composition of the plethysmograph prevents visual inspection of the inclosed tissue. With these points in mind a simple plethysmograph has been devised which eliminates all these objections.

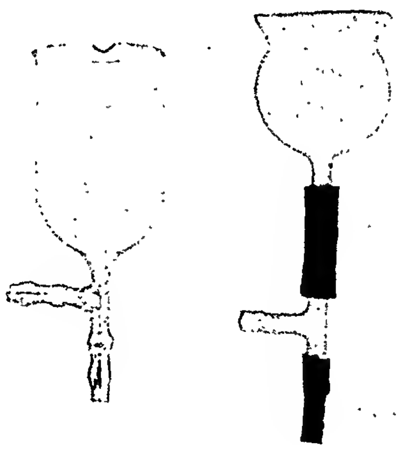


Fig. 1.

PLETHYSMOGRAPH

The plethysmograph is a cylinderical cup-shaped glass container. The bottom is entirely open and the top is closed, except for the opening of the outlet tube. The one shown on the right in Fig. 1 was made for large cats and is 40 mm. in diameter and 50 mm. high (this height is somewhat greater than actually needed). The two outlet tubes are clearly seen at the top of the picture, while at the bottom the notch intended for the passage of blood vessels is evident. This plethysmograph was made by a local glass blower. A workable, and in our experience equally satisfactory, apparatus for small cats can be made from a large thistle tube connected with a glass T-tube by means of rubber tubing (Fig. 1).

*From the Department of Physiology, University of Buffalo.

TECHNIQUE

The usual procedure of opening the abdomen and dissecting the kidney free from attachments is employed. The plethysmograph is prepared for use by placing a short rubber tube on the straight outlet arm. Through this tube, and extending through the bottom opening of the plethysmograph, a suture thread is passed. A small wire is used to pull the thread through the tube and plethysmograph. A suture needle is used to fix the lower end of this thread to the capsule of the kidney. The plethysmograph is now placed over the kidney, adjusted, and held in the desired position by means of a clamp from a base stand. The thread is pulled and the kidney is suspended in the glass chamber under as little tension as possible. A pinch clamp on the rubber

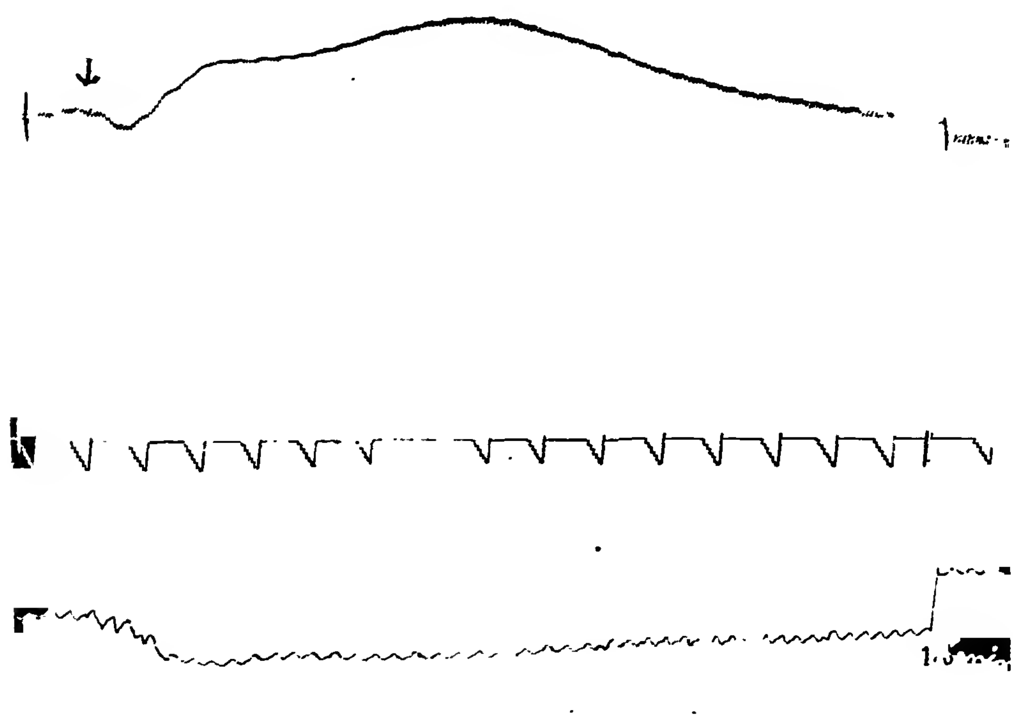


Fig. 2.—Blood pressure of a cat taken with a mercury manometer, upper tracing; base line and time in five seconds, middle tracing; and record of kidney volume, lower tracing. Note the return of the kidney volume to even less than normal during a rest period of one and one-half minutes. A single dose of 0.5 c.c. of a 1:50,000 solution of adrenalin given as indicated. Cat weight 2,000 Gm.

tubing holds the thread from slipping and closes the opening in the tubing. The kidney can be clearly seen and the plethysmograph can be adjusted so as to relieve all pressure on the vessels. A little warm mineral oil placed in the abdomen will make the plethysmograph airtight. Frequently no oil is required to make an airtight seal. If the intestines enter the plethysmograph, these may be blocked off with cotton rolled in vaseline, provided care is taken not to obstruct the veins of the kidney. The whole area is kept warm by closing the abdominal wall around and over most of the plethysmograph. The side arm of the plethysmograph is attached in the usual way to the recording tambour.

Records taken of kidney volume usually clearly show respirations and heartbeats, as well as changes in volume due to vasomotor activity. Fig. 2 shows the effect of adrenalin injected intravenously in an anesthetized cat. The expected rise of blood pressure occurs and constriction of the kidney, as indicated by the fall in the plethysmographic record, is evident.

AN ACCURATE CONSTANT TEMPERATURE WATER BATH CONSTRUCTED AT VERY LOW COST*

Benjamin C. Riggs, M.D., Philadelphia, Pa.

THE need for a small, accurate, constant temperature water bath of low cost led to the development of the design described. The accuracy was to be within 0.1° C. and the capacity 2 liters. The final cost came to less than four dollars. The uses for such a bath in the office or hospital laboratory are readily apparent and include blood amylase and lipase, trypsin, urea, amino acids, calcium, gastric enymes, inactivation of serum, and vaccine preparation.

Theory.—Essentially, the apparatus consists of an electrically powered immersion heater, controlled by a simple thermoregulator, installed in a small water bath of suitable shape to insure thorough mixing by convection.

The relation between vapor tension and temperature has long been known and practically used. In the design described, a mercury column, driven upward by the rising vapor tension of a suitable fluid subjected to rising temperature, is coupled at an adjustable point to a small mercury manometer. One arm of this manometer serves as the moving element of a single pole switch, making and breaking the power circuit of the heater. Undesirable arcing in the switch is eliminated by a low capacity condenser connected across the switch circuit.

The heating element also uses a well-known principle. The resistance to alternating current of a low concentration of electrolytes in water is used to produce high temperatures within a small space. In this design, two heavy gauge, parallel wires are immersed in the tap water of the bath and connected to either side of the 110 volt A.C. house circuit through a thermoregulator. When this is in closed position, the water between the exposed wires is kept at boiling temperature. Power consumption is regulated to suit the size and temperature of the bath by a sliding insulator covering one of the wires.

To eliminate the conventional mechanical stirrer, the heater is placed centrally in an approximately hemispherical bath, with the result that convection currents eliminate any temperature variations outside the sensitivity limits of the thermoregulator. (The presence in the bath of test tubes, flasks, etc., presents no measurable interference if the spacing between each is at least 2 cm.)

Construction.—1. Heater. (Cost of materials, approximately 50 cents.)

For temperatures up to 45° C., a single pair of parallel, 18 gauge wires, of 6 cm. exposed length, 2 mm. apart, will deliver sufficient heat to maintain

*From the Laboratory of the Germantown Hospital, Philadelphia.

Received for publication, May 19, 1942.

the temperature in 2 liters of water; the circuit will be closed not more than 50 per cent of the time. For higher temperatures or larger baths a double unit is more satisfactory: two pairs of wires are used, one pair operating continuously; the other, connected in parallel, running intermittently through the thermoregulator circuit. The construction is essentially the same in either case.

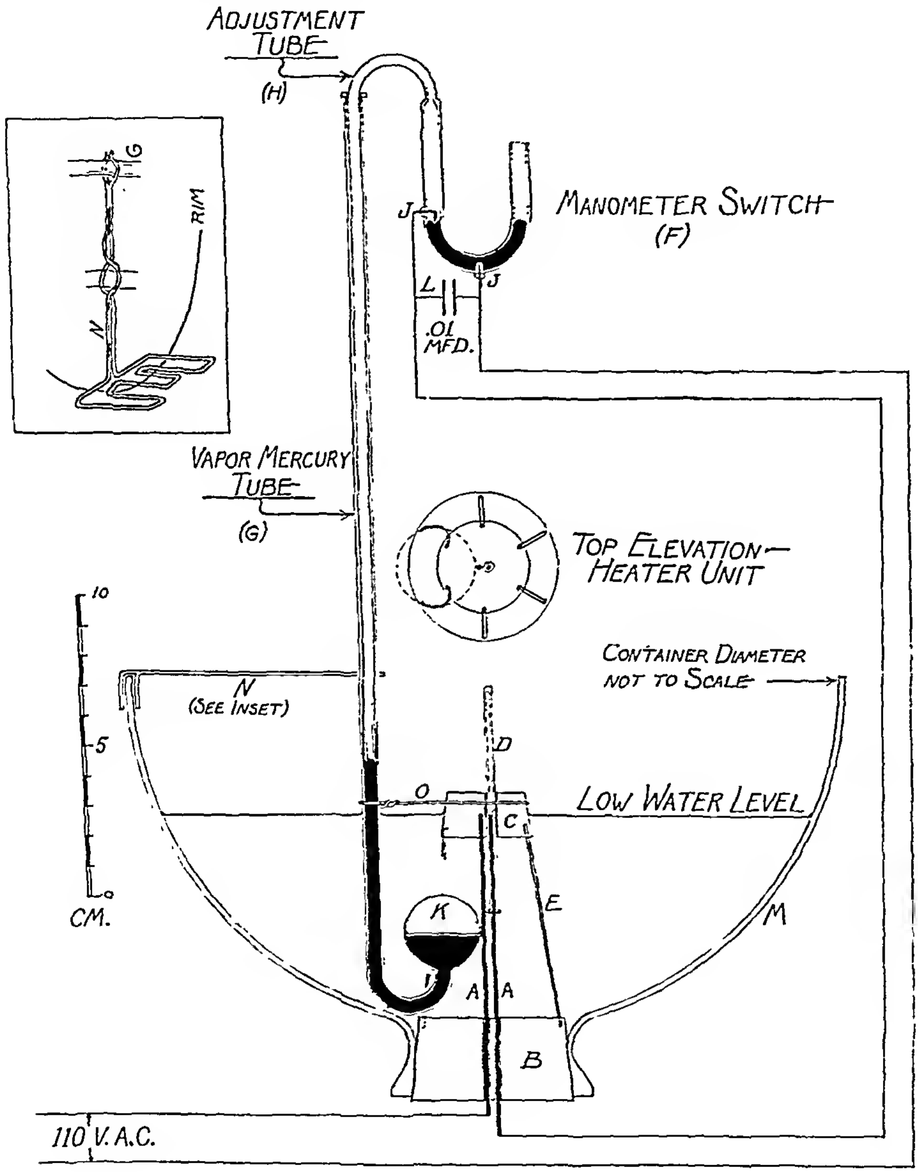


Fig. 1.

Although the apparatus now in use was built with copper heating wires, it is probable that some other metal, such as stainless steel or silver, would be less liable to the formation of objectionable salt crusts.

The heating wires (*A*) are passed through the rubber stopper (*B*) forming the base (of a size to fit tightly the neck of the container), and are secured by insertion into the rubber stopper (*C*) forming the top of the heater. A single

hole is bored in (*C*), at the insertion of one wire of each pair, to fit the glass insulating tubes, shown (*D*) in the single unit. These are lubricated with stop-cock grease; their position regulates the amount of exposed wire surface, and hence, the power consumption. A guard is built around the heating wires of painted iron wires (*E*), six in number, spaced evenly about the periphery, the ends inserted in the top and bottom stoppers. Two of these will be spread slightly to accommodate the vapor bulb (*K*) after it is constructed.

2. Thermoregulator. (Cost of materials approximately $2.50.)

Two sizes of soda lime glass tubing are used and enough "881" glass to form two beads about 2 mm. in diameter. The switch (*F*) and vapor mercury tube (*G*) have an *inside* diameter of 5 mm., and the adjusting tube (*H*) has an *outside* diameter of 4 mm. The "881" glass is used as a sealing medium around the platinum switch electrodes (*J*), because its coefficient of expansion is almost exactly that of platinum, thereby avoiding cracks. The vapor bulb (*K*) is blown to approximately 2.5 cm. diameter from the sealed end of the vapor mercury tube (*G*) *before* bending. Properly placed holes, 1 mm. in diameter, are blown in a *previously* bent section of the same sized tubing to form the manometer switch (*F*). One centimeter lengths of 20 gauge platinum wire (*J*) are sealed into these holes in beads of "881" glass. The adjusting tube (*H*) is sealed to the manometer *after* bending, and is fitted with a snug rubber washer cut from tubing to enable it to be set at any desired height within the vapor mercury tube (*G*). The 0.01 microfarad tubular condenser (*L*) is mounted between the outside ends of the electrodes to facilitate wiring and give support to the electrodes and seals. The mercury in the switch should just make thorough contact with the upper electrode, with both sides unobstructed, and the adjusting tube vertical.

The vaporizing fluid in the vapor bulb is best chosen by trial and error for the temperature range contemplated. Satisfactory results have been obtained with mixtures of ethyl ether and absolute ethyl alcohol, for temperatures from 35° C. to 45° C., and with petroleum ether from 40° C. to 60° C. The amount of fluid, size of air bubble, and amount of mercury are also best found by experiment. (The author used mercury 10 c.c. and fluid 0.5 c.c.)

The wiring should be evident from the diagram. A second heating unit is simply wired directly across the power lines. It has been found convenient to wire the manometer switch to a plug, fitting into a socket at the base of the heater, to facilitate removal for cleaning the bath.

3. Container (*M*). (Cost of materials, approximately $1.00.)

The most satisfactory container was found to be an inverted glass lamp shade of hemispherical shape, the neck receiving a No. 11 rubber stopper, the rim about 30 cm. in diameter. This pattern is easily obtainable for about $1.00 and has been perfectly suited.

The thermoregulator is so placed that the vapor bulb passes between two of the guard wires, slightly spread to receive it, and rests in contact with the heating element. (This should be the intermittent heating element if a double unit is used.) The vapor mercury tube is held vertically by a simple clamp (*N*) bent from iron wire, slipping onto the rim of the container. An auxiliary clamp

(*O*) is bent in the form of a double-ended hook to steady the bulb against the heating element. Clamp (*N*) is also bent to receive a standard, mercury immersion thermometer.

Operation.—The apparatus is designed for use on the standard, 110 volt A.C. circuit. Continuous operation requires a daily check on the water level and flushing out of the bath weekly. No other attention has been found necessary. When beginning use, the bath should be filled with water at approximately the desired temperature, and the adjusting tube raised so that the switch just makes contact. Final adjustment is postponed until the thermoregulator and bath have reached equilibrium, or about one-half hour after filling. The maximum over-all variations in temperature have not been observed to exceed 0.2° C.

SUMMARY

A new design is described for an extremely inexpensive, small, easily constructed, constant temperature water bath, for use in the hospital or office laboratory. The theory, construction, and operation are given in detail.

LEVINSON TEST FOR TUBERCULOUS MENINGITIS*

Presentation of 110 Cases and a Discussion of the Principle and Technique of the Test

Daniel Burman, A.M., M.D., and Roslyn Weintraub, B.A.
New York, N. Y.

INTRODUCTION

The Levinson test for tuberculous meningitis is a very accurate diagnostic procedure. A study of the literature and a personal survey of several hospitals and many physicians revealed that this test is little known and used still less. As a result, we feel that a presentation of the results of this test on 110 cases, a review of the results of previous investigations, and a discussion of the principle and technique will be of value and may serve to increase the use of this valuable adjunct in the diagnosis of meningitis.

HISTORY

In 1917 Tashiro and Levinson[1] published a report on the alkaloidal and metallic precipitation of cerebrospinal fluid in the diagnosis of meningitis. It was not until 1919 that this test again appeared in the literature. At that time Levinson[2] included it in his book on spinal fluid without discussing its principle. The first clinical report that we could find on this subject was the paper published by Gleisch[3] in 1932. In this paper he discusses the test and reports that in his cases the results were uniformly good. The same author[4] again published a clinical report in 1938 in which he states that 51 cases (Tables I, II,

*From the Otolaryngology and Pathology Departments of Harlem Hospital, New York, N. Y.

Received for publication, May 22, 1942.

III) were examined and tested by this method with very good results. The report showed 38 cases with a positive Levinson test and 13 cases with a negative result. Of the 38 cases, 23 revealed tuberculosis organisms; 6 showed tuberculosis at autopsy; one tuberculous patient had tuberculosis organisms in the spinal fluid; and 8 did not have tuberculosis. However, of these 8, one had an intracranial hemorrhage with multiple lacerations; 2 had cerebral hemorrhage with hypertensive cardiovascular disease; one had encephalitis lethargica; 3 were epileptics; and one had staphylococcus meningitis. All the 8 nontuberculous meningitis cases were positive, since there was either blood or serum present in the spinal fluid. Of the 13 negative tests all were nontuberculous, 10 had a meningococcic meningitis, and 3 had poliomyelitis.

TABLE I

	Positive—38	Negative—13
Tuberculous	30	0
Nontuberculous	8	13

TABLE II

Nontuberculous positives	8	
Intracranial hemorrhage		3
Encephalitis lethargica		1
Epilepsy		3
Staphylococcus meningitis		1

TABLE III

Nontuberculous meningitis	13	
Meningococcic		10
Poliomyelitis		3

In 1939 Gleisch and R. Weintraub[5] published a study of the comparative value of the Levinson and tryptophan tests in the diagnosis of tuberculous meningitis. They pointed out that the tryptophan test was markedly inferior to the Levinson test. Their results showed (Table IV) that in 18 cases of positive tuberculosis all had positive Levinson tests, but only 16 had a positive tryptophan A, 12 a positive tryptophan B, and 2 were negative in both tryptophan A and B tests. Furthermore, in 8 cases of nontuberculous meningitis with clear fluid (Table V) all had negative Levinson tests, but 8 had a positive tryptophan A and 3 had a positive tryptophan B. They also showed that in 7 cases of bloody spinal fluid and 9 cases of purulent meningitis (Table VI) all had positive Levinson and tryptophan tests; and that in 14 cases without meningitis all had negative tryptophan and Levinson tests.

In 1940 S. Weintraub[6] published a paper on the various diagnostic procedures for tuberculous meningitis. He was favorably impressed with the results of the Levinson test.

TECHNIQUE

Into each of two small test tubes of uniform width, about 0.3 cm. in diameter, place 1 c.c. of spinal fluid. Add to one of the tubes 1 c.c. of 3 per cent sulfosalicylic acid and to the other 1 c.c. of 1 per cent mercuric chloride. Stopper the tubes and allow them to stand for from twenty-four to forty-eight hours at room temperature and then measure the height of the sediments. (The very small flat-bottomed serum vials are very useful tubes to use for this test.) Normally, both are very small; in suppurative meningitis the sulfosalicylic precipitate is very heavy and the mercuric chloride precipitate is very small. In

tuberculous meningitis the opposite takes place, the sulfosalicylic acid precipitate being small and the mercuric chloride precipitate being large. In order to read a positive Levinson test the foregoing conditions must be fulfilled and the mercuric chloride precipitate must be from two to three times as great as the sulfosalicylic precipitate; that is, when measuring the height of the precipitate in the two tubes, diameters of which are the same, the height of the mercuric chloride precipitate must be two to three times as large as that of the sulfosalicylic acid. It is to be remembered that this test is only to be performed on clear spinal fluid.

TABLE IV

COMPARATIVE VALUE OF THE LEVINSON AND TRYPTOPHAN TESTS IN 18 CASES OF PROVED TUBERCULOUS MENINGITIS

	LEVINSON	TRYPTOPHAN A	TRYPTOPHAN B	TRYPTOPHAN A AND B
Positive	18	16	12	12
Negative	0	2	6	2

TABLE V

EIGHT CASES OF NONTUBERCULOUS MENINGITIS WITH CLEAR FLUID

	LEVINSON	TRYPTOPHAN A	TRYPTOPHAN B
Positive	0	8	3
Negative	8	0	5

TABLE VI

SIXTEEN CASES WITH BLOOD OR PUS IN THE SPINAL FLUID (BLOOD 7—PUS 9)

	LEVINSON	TRYPTOPHAN
Positive	16	16
Negative	0	0

FOURTEEN CASES WITH NONMENINGITIC SPINAL FLUID

	LEVINSON	TRYPTOPHAN
Positive	0	0
Negative	14	14

PRINCIPLE

The spinal fluid of tuberculous meningitis, like normal spinal fluid, has a pH of approximately 7.4 immediately after withdrawal, but ascends to 8.1 or higher on standing for from twenty-four to forty-eight hours. Spinal fluid from epidemic meningitis has a pH 7.2 to 7.5 but goes up only slightly on standing from twenty-four to forty-eight hours. The more turbid the fluid the longer it retains its acidity. An explanation that has been offered for this phenomenon is as follows: Normal spinal fluid loses H ions on standing. However, if it is tightly corked it does not change its pH. Thus, this phenomenon may be due to the loss of carbon dioxide. Tuberculous meningitic spinal fluid reacts just as the normal spinal fluid, only more rapidly. Epidemic meningitic spinal fluid changes its pH very little and becomes more acid on being corked. This may be due to lactic acid formation.

Bacteriology is helpful in making a diagnosis when the results are positive. However, it is not of much aid when negative results are obtained. Globulin is increased in all meningeal inflammations, but the amount of increase does not help to determine the disease because the ranges overlap each other.

The pH of spinal fluid changes on standing and varies between epidemic and tuberculous meningitis, as discussed above. Levinson stated in 1917: "If the difference in the reaction of the different fluids is great enough, we should

be able to distinguish their protein in a variety of ways, even if their protein concentration might be the same. The electrical charge of a protein depends on the reaction of the medium. In acid solution proteins become electropositive and in alkaline solution electro-negative. Thus one of the ways of precipitating protein is to let it combine with some radical to form an insoluble salt. From the standpoint of the electrical charge of the protein, such a protein salt is necessarily either a positive protein radical forming a protein salt with the negatively charged ions (alkaline precipitates) such as tungstic acid, picric acid, etc., or negatively charged protein combining with a positively charged metal (metallic precipitates) such as copper, silver, mercury, zinc, and lead. If these protein salts are sufficiently insoluble, the precipitate will come down."

TABLE VII

ONE HUNDRED AND TEN CASES EXAMINED BY LEVINSON TEST

Proved tuberculous meningitis		68
Positive tests	64	
Negative tests	2	
Equivocal tests	2	
Controls		33
Meningismus		
Nontuberculous meningitis		
Nonmeningitic		
Discarded cases		9
Red blood cells	6	
Quantity insufficient	3	

TABLE VIII

SIXTY-EIGHT CASES OF PROVED TUBERCULOUS MENINGITIS

Diagnosed by finding the organism in the spinal fluid or on autopsy	41
Diagnosed by clinical, x-ray, and other laboratory tests	27
Deceased	68

Cataphoresis experiments proved that the protein in epidemic meningitis moves toward the cathode, showing the presence of positively charged protein ions, whereas in tuberculous meningitis the protein ions went to the anode, proving a greater number of negatively charged protein ions in this condition. In nonmeningitic fluids there is very little precipitation in cataphoresis. Thus the pH variation is enough to produce electrical charges in the protein. Therefore, it should be possible to choose proper precipitants to distinguish the various forms of meningitis; that is, there should be more precipitate with a metallic precipitant than with an alkaloid precipitant in tuberculous meningitis, and the reverse in epidemic meningitis. Experimental work subsequently proved this to be true.

Normal fluid is only slightly turbid when sulfosalicylic acid is added and remains clear with mercuric chloride. This condition obtains only immediately after the addition of these reagents. However, on standing both form precipitates. In tuberculous meningitis the addition of sulfosalicylic acid causes the fluid to become turbid immediately whereas with the addition of mercuric chloride the fluid remains clear. In twenty-four hours sediments are found in both tubes, but the precipitate due to the mercury is two to three times as much as that due to the sulfosalicylic acid. In epidemic meningitis the sulfosalicylic acid precipitates the protein quickly and the mercuric chloride precipitates the protein slowly. In this case twenty-four hours after the addition of the pre-

cipitants the sulfosalicylic acid sediment is two to three times as great as that of the mercuric chloride. It cannot be overemphasized that the principal feature in this test is the ratio and not the total amount of precipitate which is found, and this is directly dependent upon the pH of the protein.

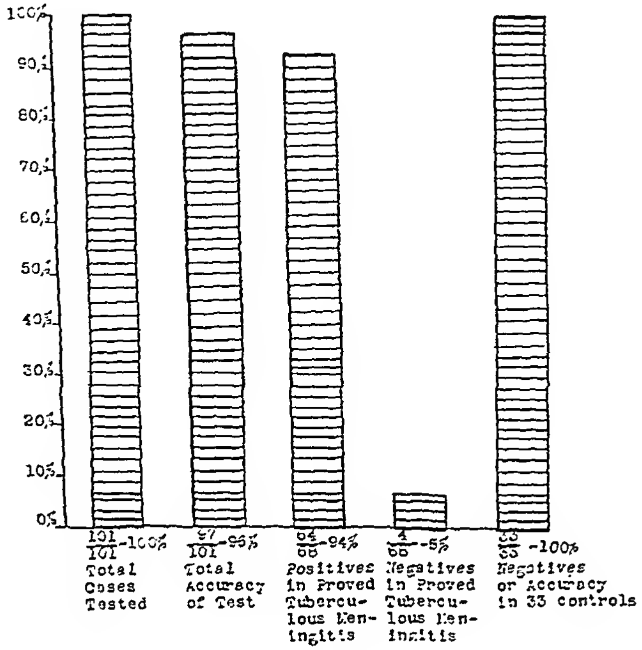


Fig. 1.

CASE STUDIES

The spinal fluids of 110 cases were examined. Nine of these had to be discarded for the following reasons: Six had red blood cells and three had insufficient fluid for a Levinson test after the other tests had been performed. As a result we have only 101 cases on which tabulations can be based. There were 68 cases of tuberculous meningitis and 33 controls. These 33 cases were diagnosed as meningismus, nontuberculous meningitis, or cases in which no meningeal signs were present. Sixty-four of the 68 cases were positive while 2 were negative and 2 gave equivocal results when tested by the Levinson method; that is, 2 could have been read either way—positive or negative. We are considering these as negative results. Thus we have 4 negative results. Of the 33 controls all gave negative results in this test.

Forty-one of the 68 cases, or 60.3 per cent, were proved to be tuberculous meningitis by one or more of the following methods: finding the organism in the spinal fluid or autopsy. The other 27, or 29.7 per cent, were diagnosed as tuberculous meningitis on the basis of clinical, x-ray, and other laboratory findings plus the fact that they all died. Thus 64/68, or 94 per cent, gave positive Levinson tests, and 4/68, or 6 per cent, gave negative Levinson tests. Furthermore, all the controls, that is, 33/33, or 100 per cent, gave negative Levinson tests; or, to express it in another way, 97/101, or 96 per cent, gave accurate results by the Levinson method.

If we now combine our results with those reported above, taken from the literature, there is a total of 217 cases. Of these, 116 were proved to be tubercu-

lous meningitis and gave 112 positive results and only 4 negatives. One hundred and three were nontuberculous and gave the following results:

Positive Levinson tests 30

All had either red blood cells or pus in the spinal fluid

Negative Levinson tests 70

These were made up of normals and cases of poliomyelitis, meningismus, nontuberculous meningitis, and 2 equivocal cases.

Quantity Insufficient for a Levinson test 3

After all the routine tests were performed, there was not sufficient spinal fluid left for the performance of the Levinson test.

COMMENT

We have presented 217 cases which were studied for the determination of the accuracy of the Levinson test. Of these, 110 were our own and 107 were from the literature. Our results revealed that this test is 94 per cent accurate, whereas the total of our cases plus those of the other investigators (who found 100 per cent accurate results) brings the percentage of accuracy up to 97. As this compares favorably with any other diagnostic laboratory procedure, and as the number of cases studied is not too small, we feel that this simple laboratory test should be used more often in the diagnosis of meningitis.

It should be evident from this paper that the test is of value only in cases of meningitis where the spinal fluid is clear and where no serum has been administered, since pus, red blood cells, and serum render the spinal fluid more alkaline and result in a false positive.

SUMMARY

1. The history and a summary of the literature on the Levinson test are presented.

2. A description of the technique and the principle on which the test is based are given.

3. A total of 110 new cases and 107 cases in the literature on which the Levinson test was performed, together with a breakdown of the results to determine the accuracy of the test, is presented.

4. Tables showing the test from 94 to 97 per cent accurate in diagnosing tuberculous meningitis are included.

5. A bibliography of all the available literature on this subject is presented.

REFERENCES

1. Tashiro, Shiro, and Levinson, A.: Alkaloidal and Metallic Precipitation of Cerebrospinal Fluid in the Diagnosis of Meningitis, J. Infect. Dis. 21: 571, 1917.
2. Levinson, Abraham: Cerebrospinal Fluid in Health and Disease, 1919, page 193.
3. Gleisch, Morris: Levinson Test in Tuberculosis Meningitis, Am. J. Dis. Child. 43: 1077, 1932.
4. Gleisch, Morris: Levinson Test in Tuberculosis Meningitis, Am. Rev. Tuberc. 38: 239, 1938.
5. Gleisch, Morris, and Weintraub, Roslyn: Comparative Value of the Levinson Test, Arch. Pediat. 56: 749, 1939.
6. Weintraub, Solomon: Laboratory Procedures in Tuberculous Meningitis, Bull. Am. Acad. Tuberc. Physicians No. 5, pages 13-18, February, 1941.

667 Madison Avenue
New York, N. Y.

CHEMICAL

THE DETERMINATION OF TOTAL CHOLESTEROL AND CHOLESTEROL ESTERS IN BLOOD*

S. L. Leiboff, New York, N. Y.

METHODS for the quantitative determination of cholesterol esters in blood have been devised by Bloor and Knudsen,[1] Sperry,[2] Kelsey,[3] and others. In all these methods the free cholesterol is separated from the esters by precipitation with digitonin. For the quantitative estimation of both free and combined cholesterol, practically all methods utilize the Liebermann-Burchard color reaction.

Various procedures are used for extracting the cholesterol from blood. Bloor[4] extracts it from wet blood with a mixture of alcohol and ether. Myers and Wardell[5] dry blood with plaster of Paris and extract with chloroform. I[6] dry the blood on fat-free filter paper and extracts with chloroform.

Because of the difficulties inherent in these methods, requiring a great deal of time and painstaking manipulation, a study was undertaken to determine the conditions best suited to produce the quickest and most accurate results. This study culminated in the development of an extremely simple and accurate method for the determination of total cholesterol and cholesterol ester content in blood.

PRINCIPLE OF METHOD

Oxalated blood is mixed well with anhydrous sodium sulfate and extracted with chloroform for twenty minutes in a graduated test tube attached to an air-cooled condenser. The chloroform extract is filtered and two aliquot portions are removed. One portion is treated directly with acetic anhydride and sulfuric acid for the total cholesterol content. The second portion is used for the determination of esters as follows: The chloroform is removed by evaporation and substituted by petroleum ether from which the free cholesterol is removed with digitonin. The petroleum ether is evaporated, the esters are taken up in chloroform, and the color is developed with acetic anhydride and sulfuric acid. Both fractions are determined colorimetrically against a standard containing a known amount of cholesterol.

Preparation of Reagents.—It is important that the reagents used in the test be of the highest purity; this applies particularly to the chloroform and acetic anhydride. The following methods for the purification of these reagents have proved very satisfactory.

Purification of Chloroform.—Into a 2,000 c.c. separatory funnel introduce 1,000 c.c. of chloroform and 100 c.c. of concentrated sulfuric acid. Shake at intervals during twenty-four hours. Remove the sulfuric acid and wash the

*From the Laboratory of Lebanon Hospital, New York.
Received for publication, March 15, 1941.

chloroform with a slow stream of running water passing through a glass tube reaching to the bottom of the separatory funnel until neutral to litmus. Transfer the chloroform to a distilling flask and add 50 Gm. of anhydrous calcium chloride. Shake at intervals during twenty-four hours and distil, discarding the first and last 50 c.c. of chloroform. Transfer the distilled chloroform to an amber bottle and add 0.7 c.c. of absolute alcohol per 100 c.c. of chloroform and a few grams of anhydrous calcium chloride. The alcohol acts as a preservative. Keep the bottle tightly stoppered.

Purification of Acetic Anhydride.—Heat 500 c.c. of acetic anhydride with 50 Gm. of calcium oxide in a distilling flask under a reflux condenser for three hours. Allow to stand overnight and distil slowly. Keep in brown bottle with tightly fitting glass stopper.

Preparation of Digitonin Reagent.—In a small flask place 1 Gm. of digitonin, 10 c.c. of methyl alcohol, and 10 c.c. of distilled water. Place the flask in a beaker containing water heated to about 60° C. and rotate the flask until the digitonin is completely dissolved.

On standing the digitonin separates out of solution. It is, therefore, necessary to redissolve it by placing in hot water before use. It redissolves very readily.

Preparation of Cholesterol Standard.—Prepare a stock solution by dissolving 0.160 Gm. of pure cholesterol in 100 c.c. of chloroform. A working standard is prepared by diluting 5 c.c. of the stock standard with 95 c.c. of chloroform. Five cubic centimeters of the working standard contain 0.4 mg. of cholesterol. The standards are kept in the refrigerator in amber bottles.

Extraction Apparatus (Fig. 1).—The apparatus consists of a graduated glass tube 20 by 150 mm. The graduation marks start at zero about 40 mm. from the bottom of the tube and continue upward to a mark of 60. These graduation marks do not represent specific volumes but are used to indicate the chloroform level in the tube. The inside mouth of the tube is ground to fit a coiled glass air-cooled condenser. The whole length of the condenser is 260 mm. and the coiled portion consisting of six coils is 100 mm. long and 10 mm. wide. This condenser is a modification of the one suggested by Breh.[7]

The glass rod is 220 mm. long and 5 mm. in diameter.

PROCEDURE

Determination of Total Cholesterol.—When a determination of the total cholesterol only is required, the following procedure is to be followed:

Place 0.5 c.c. of oxalated blood into the extraction tube and add approximately 2 Gm. of anhydrous sodium sulfate and exactly 10 c.c. of chloroform. Mix with a glass rod to a homogeneous mixture by pressing and rubbing the rod against the walls of the tube. Hold up the extraction tube in a perfectly vertical position level with your eye, and without removing the rod, read the graduation mark at the meniscus of the chloroform. Attach the condenser to the tube without removing the rod, and heat gently over a microburner or an electric heater for ten minutes. Allow to cool for a few minutes, detach the condenser, and stir up the powder thoroughly with the rod. Attach the condenser and heat for ten more minutes to complete the extraction. Allow to cool to room temperature and add chloroform to the original graduation mark. Mix well with

the rod and filter through a very small piece of absorbent cotton packed somewhat tightly in the stem of a small glass funnel. Keep the funnel covered with a watch glass to prevent evaporation.

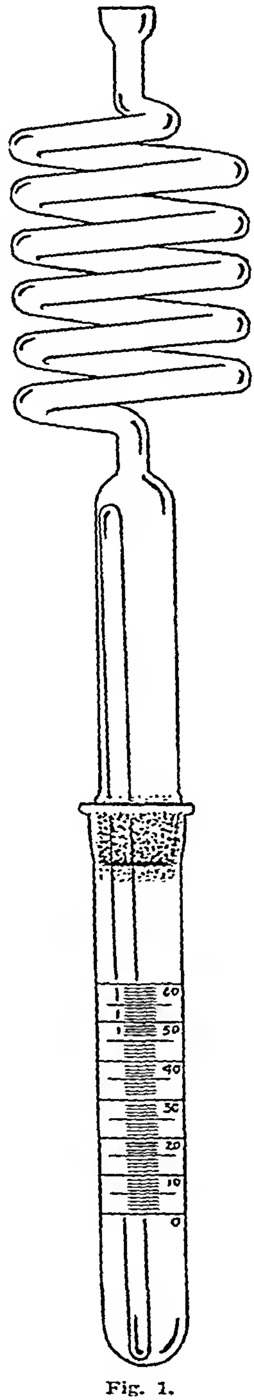


Fig. 1.

Transfer 5 c.c. of the chloroform extract to a test tube. In another test tube place 5 c.c. of the working standard (equal to 0.4 mg. cholesterol). Place the standard and the unknown in a beaker of water kept between 20° and 25° C.

Add to each tube 2 c.c. of acetic anhydride and 0.1 c.c. of concentrated sulfuric acid. Cover the tubes with clean dry corks, mix contents, and set aside in a dark place for twenty minutes. Compare colors in the colorimeter by setting the standard at 15 mm.

Calculation of Result.—

$$\frac{2400}{X} = \text{Milligram of cholesterol per 100 c.c. of blood.}$$

$$X = \text{Reading of the unknown.}$$

Determination of Total Cholesterol and Cholesterol Esters.—Place 1.5 c.c. of oxalated blood into the extraction tube and add approximately 6 Gm. of anhydrous sodium sulfate and exactly 15 c.c. of chloroform. Mix well with the rod and note the graduation mark at the meniscus of the chloroform. Extract for twenty minutes and filter through absorbent cotton, following all the steps already described under the determination of total cholesterol.

Transfer 2.5 c.c. of the chloroform to a test tube and add 2.5 c.c. of chloroform. Stopper with a clean cork and set aside for the determination of total cholesterol.

Transfer 7.5 c.c. of the chloroform extract into a test tube (20 × 150 mm.). This is for the determination of cholesterol esters. Place this tube in a water bath heated to about 70° C. until all the chloroform is driven off. Cool to room temperature and add 9 c.c. of petroleum ether (B. P. 30° to 60° C.) and 0.1 c.c. of digitonin solution previously heated to put in solution. Cork the tube and shake well; the cholesterin digitonide is precipitated immediately. Allow to stand for a few minutes and filter through a small piece of cotton tightly packed in the stem of a small funnel. In this case the cotton has to be packed very tightly so that no particles of digitonide can pass through.

Remove 6 c.c. of the petroleum ether filtrate into a similar large test tube and place in the water bath at about 60° C. until all the petroleum ether is completely evaporated. Allow the tube to cool to room temperature and add 5 c.c. of chloroform. This contains the cholesterol esters only.

Into a third tube place 5 c.c. of the cholesterol working standard.

Place the two unknowns and the standard in a beaker of cold water at a temperature between 20° and 25° C., and to each tube add 2 c.c. of acetic anhydride and 0.1 c.c. of concentrated sulfuric acid. Mix the contents and put the beaker in a dark place for twenty minutes and compare in the colorimeter with the standard set at 15 mm.

Calculation of Results.—

Total Cholesterol: 2,400 divided by reading of unknown
= milligrams per 100 c.c. of blood.

Cholesterol Esters: 1,200 divided by reading of unknown
= milligrams per 100 c.c. of blood.

Precautions.—The color developed by the action of acetic anhydride and sulfuric acid on cholesterol changes rapidly on removal from the water bath and on exposure to light. Since this change is at a different rate in the standard and in the blood extracts, it is important that the colorimetric matchings be made

as rapidly as possible after removal from the dark. It should not exceed three minutes. When a large number of cholesterol determinations are to be performed simultaneously, it is advisable to set up a number of standards and use a new standard every three minutes. Each standard is removed from the beaker simultaneously with the unknowns to be read. As this reaction is also seriously affected by water, it is important that all glassware used in this test be perfectly dry.

DISCUSSION

A number of experiments were conducted to check the accuracy of the method. The first step was to determine whether this method of extraction removes all the cholesterol from the blood. A series of extraction tubes were set up with 0.5 c.c. of blood in each. Cholesterol in increasing amounts were added to each successive tube. As is shown in Table I, the added cholesterol was completely recovered within experimental error.

TABLE I

RECOVERY OF ADDED CHOLESTEROL FROM BLOOD

TUBE	ORIGINAL CHOLESTEROL CONTENT IN 0.5 C.C. OF BLOOD (MG.)	ADDED CHOLESTEROL (MG.)	CHOLESTEROL IN EACH TUBE (MG.)	CALCULATED AMOUNT OF CHOLESTEROL IN 100 C.C. OF BLOOD (MG.)	AMOUNT OF CHOLESTEROL RECOVERED (MG.)
1	0.88	None	0.88	176	176
2	0.88	0.4	1.28	256	249
3	0.88	0.8	1.68	336	330
4	0.88	1.2	2.08	416	422
5	0.88	1.6	2.48	496	487
6	0.88	2.0	2.88	576	565

In another experiment cholesterol equivalent to 1,200 mg. per 100 c.c. of blood was dissolved in petroleum ether and precipitated with digitonin. The petroleum ether filtrate was evaporated and replaced by chloroform. Treatment with acetic anhydride and sulfuric acid failed to develop any trace of color, thus showing that the digitonin solution as used in this method completely precipitates free cholesterol.

RECOVERY OF CHOLESTEROL ESTERS

In this experiment cholesterol esters in increasing amounts were added to blood. The cholesterol esters were extracted from 100 c.c. of pooled blood mixed in a large flask with anhydrous sodium sulfate and extracted with chloroform.

TABLE II

RECOVERY OF ADDED CHOLESTEROL ESTERS FROM BLOOD

TUBE	ORIGINAL ESTER CONTENT IN 1.5 C.C. OF BLOOD (MG.)	ADDED ESTERS (MG.)	TOTAL ESTERS (MG.)	CALCULATED AMOUNT OF ESTERS PER 100 C.C. OF BLOOD (MG.)	CHOLESTEROL ESTERS RECOVERED PER 100 C.C. OF BLOOD (MG.)
1	0.93	None	0.93	62	61
2	0.93	0.5	1.43	95	94
3	0.93	1.0	1.93	129	131
4	0.93	3.0	3.93	262	256
5	0.93	5.0	5.93	395	388

The free cholesterol was completely removed with digitonin, and the cholesterol esters were dissolved in chloroform. Fifty-seven milligrams of cholesterol esters were obtained, as shown by a quantitative estimation. Varying amounts of these cholesterol esters added to samples of blood were completely recovered. This is shown in Table II.

More than four hundred determinations were performed by this method on blood coming to the laboratory for routine chemical examination. The average figure for total cholesterol in normal blood was about 180 mg., and the average ester content about 65 mg. per 100 c.c. of whole blood.

SUMMARY

A simple and accurate method for the determination of total cholesterol and cholesterol esters in blood is described. The blood is mixed with anhydrous sodium sulfate and extracted with chloroform. One aliquot portion of this extract is used for the determination of cholesterol esters after removing the free cholesterol with digitonin. The extracts are treated with acetic anhydride and sulfuric acid and compared colorimetrically with a standard cholesterol solution.

REFERENCES

1. Bloor, W. R., and Knudsen, A.: The Separate Determination of Cholesterol and Cholesterol Esters in Small Amounts of Blood, J. Biol. Chem. 27: 107, 1916.
2. Sperry, W. M.: The Relationship Between Total and Free Cholesterol in Human Blood Serum, J. Biol. Chem. 114: 125, 1936.
3. Kelsey, F. E.: Determination of Cholesterol, J. Biol. Chem. 127: 15, 1939.
4. Bloor, W. R.: The Determination of Cholesterol in Blood, J. Biol. Chem. 24: 227, 1916.
5. Myers, V. C., and Wardell, E. L.: The Colorimetric Estimation of Cholesterol in Blood, With a Note on the Estimation of Coprosterol in Feces, J. Biol. Chem. 36: 147, 1918.
6. Leiboff, S. L.: A Simplified Method for Cholesterol Determination in Blood, J. Biol. Chem. 61: 177, 1924.
7. Breh, F.: The Use of Air-Cooled Condensers in Blood Cholesterol Determinations, J. Lab. & Clin. Med. 24: 635, 1939.

MICROCHEMICAL DETERMINATION OF YELLOW PHOSPHORUS*

LIEUT. SIDNEY KAYE,† U. S. ARMY

PHOSPHORUS is one of the more commonly known elements. It is, therefore, fitting and important that accurate, specific, and sensitive methods for its detection and determination should be available. The literature describes several satisfactory procedures based on gravimetric, volumetric, and colorimetric analyses. A new method for the confirmation and quantitative determination is herein described, using a spot test color reaction which is confined to a standard area the color intensity of which is proportional to the concentration of phosphorus present. This new method is not only specific and sensitive but also very simple. Its principle is based upon the formation of phosphine which in turn easily reduces a mercuric bromide test paper, yielding a canary yellow color.

Phosphorus may exist in several forms; the best known of these are yellow and red. Red phosphorus is considered by most authorities as nontoxic, whereas yellow phosphorus is very toxic and 0.1 Gm. has been recorded to be fatal. The method outlined here is applicable to yellow phosphorus only and is satisfactory, since our interest is primarily from a toxicologic viewpoint. Yellow phosphorus is used commonly in rat poison pastes and acute poisoning is usually accidental, but suicides and homicides have been recorded wherein phosphorus had been used.

Phosphorus may remain in the system for several days, but the greater the delay in obtaining the specimen, the less is the possibility of detecting it. The procedure of isolating it for analysis depends upon its volatility. Stomach contents are best suited for analysis.

A brief summary of the toxicology of phosphorus is included in this paper for completeness.

Symptoms.—Progressive pain and burning along the gastrointestinal tract, with belching, choking, vomiting, and diarrhea are present. The following additional symptoms are frequently noted: garlic odor on the breath, jaundice of the skin, bleeding from the nose and intestines, muscular paralysis and impairment of the special senses. Terminally, stupor, delirium, and coma occur. Death occurs usually within two to six days.

Treatment.—Wash the stomach with 1 per cent potassium permanganate and large amounts of suspended charcoal. If done quickly, this may be effective.

Post-Mortem Pathology.—There is corrosion of the mucous membrane of the gastrointestinal tract, with fatty degeneration and acute yellow atrophy of the

*From the Puerto Rican Department Laboratory, San Juan, Puerto Rico, Lieutenant Colonel V. H. Cornell, M.C., commanding.

†First Lieutenant, Sanitary Corps.

Received for publication, May 16, 1942.

liver. The stomach contents, as a rule, have the odor of garlic, and if viewed in the dark, give off a greenish phosphorescent light.

Chemical Analysis.—The following qualitative tests have been used to identify phosphorus positively:

1. Odor: The garliclike odor may be recognized on opening the stomach.

2. Scherer's test: A portion of the finely divided material is placed in an Erlenmeyer flask and suspended in distilled water. Two strips of filter paper are suspended over the suspected sample, one impregnated with silver nitrate and the other with lead acetate. The contents are heated at about 50° C. on a water bath for approximately an hour. If the silver nitrate paper turns black, either phosphorus or hydrogen sulfide may be present. If both papers turn black, hydrogen sulfide is present and phosphorus may also be present. The fact that the silver nitrate paper turns black is not conclusive proof of the presence of phosphorus, since any volatile reducing agent, such as formaldehyde, may also give the same reaction. Therefore, this test is more important in indicating the absence of phosphorus rather than its presence.

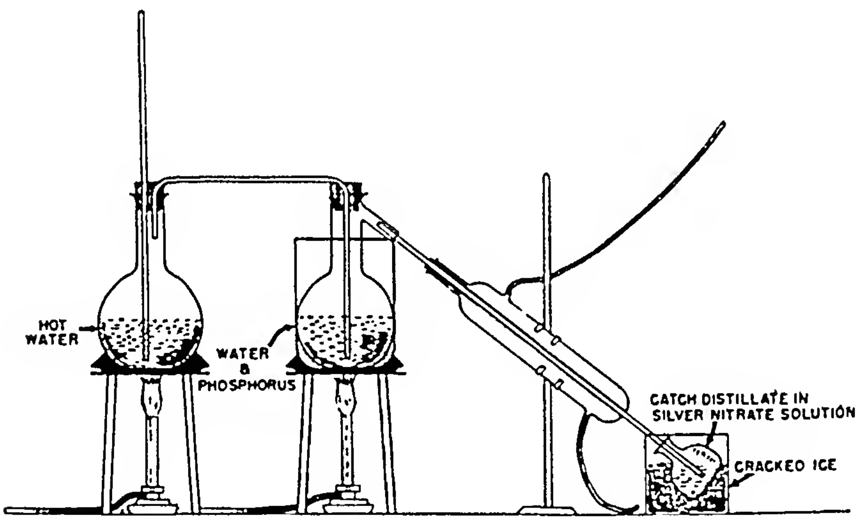


Fig. 1.

A NEW QUANTITATIVE TEST FOR PHOSPHORUS

A weighed aliquot of the sample is transferred to a steam distillation apparatus with about 100 ml. of water and an ice-cooled receiver is used to collect the distillate, as illustrated in Fig. 1. The light from the Bunsen burner is shielded by a sheet of asbestos to prevent any interference in the visual detection of the phosphorescence emitted. The distillation is performed in the dark, and the distillate is collected in a small volume of 0.5 N silver nitrate, which is cooled in a 125 ml. Erlenmeyer flask immersed in an ice bath. Greenish phosphorescence* may be seen as the phosphorus vapors reach the condenser. As little as 60 gamma† in 200 Gm. of material give a good phosphorescence. However, in the presence of alcohol, ether, benzene, turpentine, phenols, chloroform, essential oils, oxidizing agents, mercury, copper, and silver there may be a marked

*Mitscherlich's phosphorescence test.
†One gamma = 0.000001 Gm. or 0.001 mg.

diminution or complete absence of the phosphorescence. The distillation is continued until about 75 ml. are collected. As the first traces of phosphorus come over into the silver nitrate solution, a black precipitate of silver phosphide forms. After about 75 ml. have been collected, it will be noticed that on continued distillation no further precipitate forms. Silver phosphide is a heavy precipitate and settles rapidly. The supernatant liquid is siphoned off without disturbing the precipitate, leaving a small amount of liquid behind. The precipitate is then washed in the flask with several portions of distilled water to remove the last traces

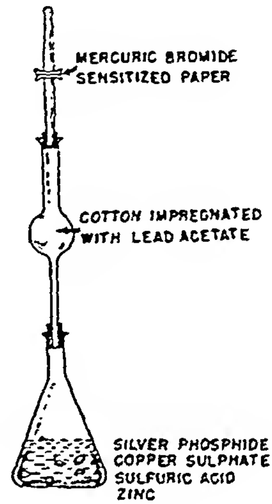


Fig. 2.

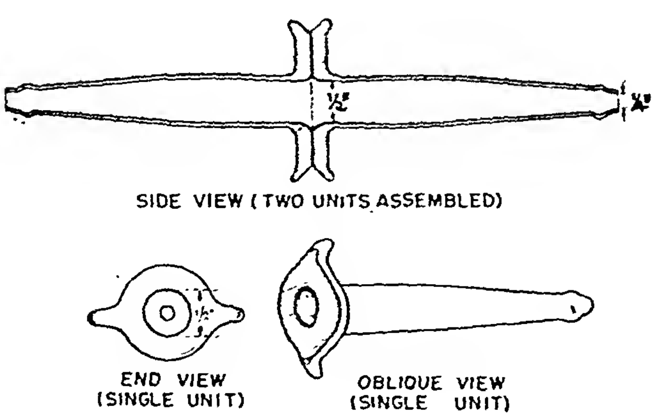


Fig. 3.

of any adhering silver nitrate. The supernatant fluid is again siphoned off. Two washings usually suffice. The washings are tested for silver ions by a chloride solution. If the precipitate is scant in the receiving vessel, make up volume to 50 ml. with water. If the precipitate is heavy, dilute to a larger known volume with water. Swirl the solution well so as to divide evenly the precipitate, withdraw a recorded small aliquot portion, and add it to a previously prepared modified Gutzeit apparatus (Fig. 2). This is essentially a 125 ml. Erlenmeyer flask containing some arsenic-free zinc, several drops of 5 per cent copper sulfate solution to

activate the process, and 25 to 40 ml. of 20 per cent sulfuric acid. The amount of sulfuric acid used should be sufficient to dissolve the precipitate in the aliquot added; excess does not interfere. The stopper in the flask supports a small calcium chloride drying tube into which is plugged some cotton impregnated with lead acetate. Above this tube is attached a set of flanges* between which is placed a disk of mercuric bromide† sensitized filter paper. On contact of the silver phosphide with the hydrogen generating solution, phosphine is liberated. The generating flask is kept warm on a water bath at about 50° C. Phosphine travels upward and is purified from hydrogen sulfide by passage through the lead acetate cotton. On reaching the flanges when it contacts the moistened mercuric bromide paper, a yellow color results which is directly proportional to the phosphine produced. Since the bores of the several flanges are uniform, comparisons can be made with phosphorus standards prepared in a similar manner. This color reaction is sensitive to less than 10 gamma of phosphorus. To be certain that the zinc and other reagents are arsenic and phosphorus free before adding the silver phosphide, hydrogen is liberated for some time and then the filter disk is examined. If this proves negative, the silver phosphide is then added and the flanges are put in place. The generator is allowed to run for about half an hour. The yellow color standards, when once prepared, are fairly stable and can be kept for several months if dry.

Other Uses for the Microchemical Flange.—The flanges described promise to be of great value for quantitative determinations in both gas and solution analyses where the intensity of a color reaction, which is proportionate to known amounts of the substance concerned, can be recorded.

Their use is being studied for the determination of sulfides, cyanides, alcohol, phenol, and other volatiles. For substances in solution which give brilliant graded colors with a detector, the flanges also have proved successful. A rapid method for the detection of lead in urine has been perfected and will be published later. The flange device was used in the Chemical Laboratory at Bellevue Hospital, New York City, while seeking more accurate and sensitive methods for the determination of arsenic. A paper on the subject is in progress and will be published from Bellevue Hospital.

Its broader application has been successfully demonstrated in the past few months at the Puerto Rican Department Laboratory.

SUMMARY

A brief review of the toxicology of phosphorus poisoning is given.

A new specific method for the quantitative microdetermination of phosphorus is described.

The principle involved depends upon trapping the phosphorus as silver phosphide, generating the phosphine by hydrogenation, and measuring the phosphine generated on a mercuric bromide filter disk. The color developed is a

*The flanges may be purchased cheaply from Eck & Krebbs or E. Machlett & Sons, both of New York City.

†Mercuric bromide sensitized paper is prepared by cutting round disks of Whatman No. 40 filter paper which are impregnated with 5 per cent mercuric bromide in 95 per cent alcohol for about five minutes. After shaking the droplets off, a disk is fastened between the flanges with a rubber band.

measure of the amount of phosphorus present and is determined by comparison with standards similarly prepared.

The microchemical flanges are a new device which may be used in a similar manner for other determinations, some of which have been mentioned.

REFERENCES

1. Wolf, L., Dusing, W., and Martes, A.: Phosphorus, Mikrochem. 18: 185, 1935.
2. Bamford, Frank: Poisons, New York, 1941, The Blakiston Co.
3. Goldbaum, Leo and Umberger, Joseph: New York, Personal communications.
4. Thorne, P. C. L., and Ward, A. M.: Fritz Ephrain Inorganic Chemistry, New York, 1939, Nordemann Publishing Co., Inc.

QUALITATIVE STUDIES OF THE BILIRUBIN IN BODY FLUIDS*

II. A Comparison of the Direct Diazo Reaction by the Photoelectric Colorimeter, the Three Test Tube Method, and the Oxidation Test in Xanthochromic Spinal Fluid

Georg Lepehne, M.D., Boston, Mass.

IN A PRECEDING paper[1] qualitative studies of the bilirubin in blood serum by comparison of the direct diazo reaction using the photoelectric colorimeter, the three test tube method, and a new qualitative oxidation test for differentiation of the various types of jaundice were reported. In this paper similar studies concerning the bilirubin of xanthochromic spinal fluid will be presented.

METHOD

The xanthochromic spinal fluid was examined by the methods described in the preceding paper: the direct diazo three test tube method with use of caffeine-sodium salicylate,[2] the direct diazo photoelectric colorimeter method of Malloy and Evelyn,[3] and the qualitative oxidation test tube method[1] using a reagent consisting of nitric acid and sodium nitrite.

RESULTS

In 8 specimens of xanthochromic spinal fluid (hemorrhages, tumor, operation of the brain) the direct diazo photoelectric examination and the three test tube examination were done. In addition 7 specimens were examined by the three test tube method alone. In all 8 specimens the photoelectric direct diazo curves showed a definitely delayed, very slow ascending type of curve. On two specimens with the total bilirubin content of 2.28 mg. per 100 c.c. and 3.79 mg. per 100 c.c., respectively, in which the three test tube method revealed a biphasic-delayed direct diazo reaction, curves of delayed type were obtained too (see Fig. 1). These curves are very similar to those found in sera with delayed direct diazo reaction (hemolytic jaundice, jaundice of the newborn, etc.).[1] They are in clear contrast to the prompt direct curves obtained in serum of obstructive or

*From the Department of Medicine, Boston University School of Medicine.
Received for publication, August 13, 1942.

catarrhal jaundice. The indirect diazo reaction following the addition of methyl alcohol gave the usual steeply ascending curves (see Fig. 1). In order to express the significance of the direct curves in ratios the one-minute and the thirty-minute readings of the direct curves were compared with each other and with the one-minute and with the thirty-minute values of the indirect curves, as in the first paper (see Table I). On the average, the direct curve reached in one minute was 15.4 per cent, in thirty minutes, 36.2 per cent only of the total bilirubin content. The corresponding ratios in sera with delayed direct diazo reaction reported in the preceding paper were very similar, while the ratios in the prompt reacting sera of obstructive and catarrhal jaundice were entirely different.

TABLE I

COMPARISON OF QUALITATIVE AND QUANTITATIVE REACTIONS XANTHOCHROMIC SPINAL FLUID

NO.	TOTAL PROTEIN (MG. PER 100 C.C.)	TOTAL BILIRUBIN (MG. PER 100 C.C.)	1 MIN. DIRECT/ 30 MIN. INDIRECT (%)	1 MIN. DIRECT/ 1 MIN. INDIRECT (%)	1 MIN. DIRECT/ 30 MIN. DIRECT (%)	30 MIN. 30 MIN. DIRECT/ INDIRECT (%)
1	168	0.4	20.5	22.7	55.4	37.0
2	219	0.6	17.7	20.5	43.9	40.9
3	570	0.8	12.5	18.2	39.5	31.3
4	116	0.4	12.2	14.6	37.4	32.7
5	192	0.6	27.1	35.5	54.0	50.1
6	264	1.0	18.7	29.5	26.0	48.0
7	1,500	2.3	10.1	15.3	30.7	32.9
8	710	3.8	4.3	6.1	26.9	16.7
Average			15.4	20.3	39.2	36.2

The three test tube direct diazo reaction was carried out in 15 specimens of xanthochromic spinal fluid, and in 13 specimens the reaction was definitely delayed. The first slight redness developed in 10 cases within an average time of five and one-half minutes. In 5 cases the red color had not developed within one hour. The change to purple color, described in the first communication, was not seen. The longest period of observation was two and one-half hours. The two specimens with the high bilirubin level (Table I, Nos. 7 and 8) showed by this method, in contrast to the delayed photoelectric curves, a biphasic-delayed direct diazo reaction. Immediately after addition of the diazo reagent a trace of redness developed, but the red color increased very slowly. The comparison of the three test tube method with the photoelectric method shows that the photoelectric readings are higher than one should expect according to the very slow development of the red color in the test tube method. This fact seems to depend on the greater accuracy of the photoelectric method to detect the color.

Vaughan and Hubbard[4] claim that every xanthochromic spinal fluid gives a "prompt" direct diazo reaction when the bilirubin content exceeds 0.3 mg. per 100 c.c. But one must consider that their expression "prompt" direct diazo reaction includes also biphasic-prompt and biphasic-delayed direct reactions[1] according to the usual nomenclature, so that their results are confusing. In our specimens a really prompt direct diazo reaction was not observed by our methods in spite of the fact that the bilirubin content was higher than 0.3 mg. per 100 c.c. (Table I). These authors also believe that the type of the direct diazo reaction in xanthochromic liquor with a bilirubin content below 0.3 mg.

per 100 c.c. depends upon the total protein content. In our cases with higher bilirubin values the total protein content ranged from 49 mg. to 3,720 mg. per 100 c.c. and did not influence the type of the reaction.

The oxidation test was made in 14 specimens of xanthochromic spinal fluid. In 12 cases the test was "negative" (a straw color developed or the color did not change at all). In the two specimens with higher bilirubin content, which gave a biphasic-delayed direct diazo reaction, the oxidation test was "diminished-delayed" (a slight yellowish-green color appeared twenty to thirty seconds after addition of the reagent). In order to control the oxidation test a drop of serum of obstructive jaundice was added to a specimen of colorless liquor. A real green color developed immediately ("prompt" oxidation test).

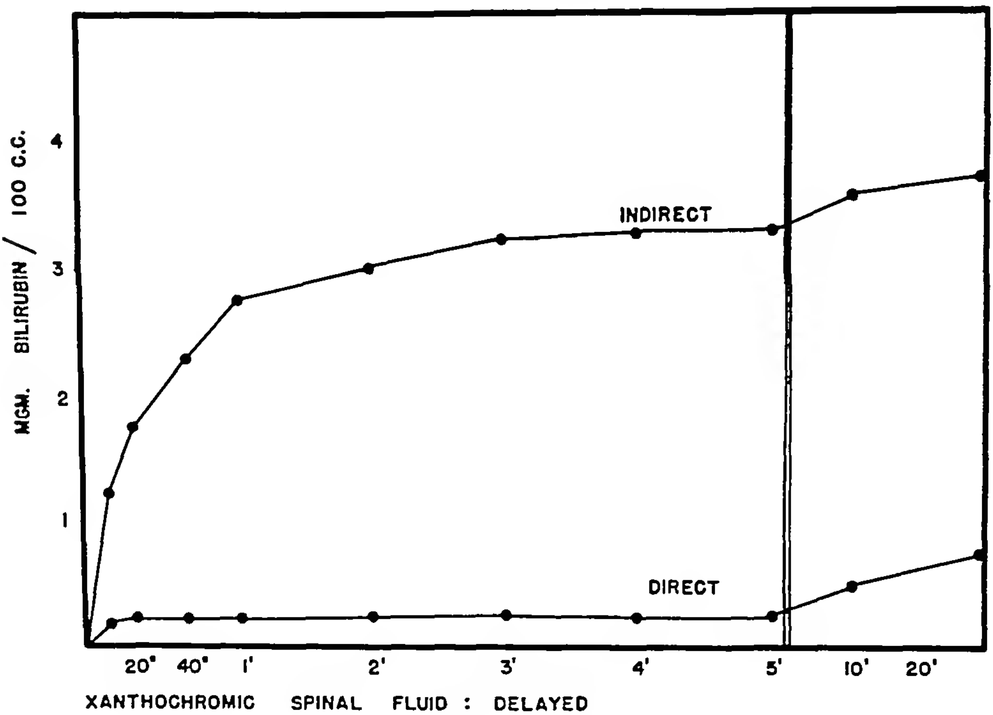


Fig. 1.—"Delayed direct diazo reaction" in a case of xanthochromic spinal fluid.

DISCUSSION

These examinations reveal that the bilirubin in xanthochromic spinal fluid gives the same qualitative reactions as the bilirubin in sera of hemolytic jaundice, of jaundice of the newborn, and of the nonhemolytic familial jaundice. These results support the theory that bilirubin giving such reactions is of anhepatic origin. Bilirubin in the blood of this type apparently did not pass the excreting liver cells. The liver cells change the direct diazo reaction from delayed to prompt (van den Bergh,[5] Aschoff,[6] McNee,[7] Lepehne,[8] Eppinger,[9] and other authors). The described examinations also reveal, as did the examinations on jaundiced serum,[1] that even in xanthochromic spinal fluid a really "negative" direct diazo reaction does not exist, when observation time is long enough. The direct diazo reaction can be, generally speaking, either prompt, biphasic, or delayed, but not negative.

SUMMARY

The bilirubin of xanthochromic spinal fluid was examined by the direct diazo three test tube method, by the direct diazo photoelectric method, and by the oxidation test. The direct diazo reaction was delayed or biphasic-delayed, and the oxidation test negative or diminished-delayed. The total protein content did not influence the type of the reaction. This bilirubin is of the same type as that in the blood serum in hemolytic jaundice and jaundice of the newborn.

REFERENCES

1. Lepehne, Georg: Qualitative Studies of the Bilirubin in Body Fluids. I. A Comparison of the Direct Diazo Reaction by the Photoelectric Colorimeter, the Three Test Tube Method and the Oxidation Test in Serum, J. Lab. & Clin. Med. 27: 1447, 1942.
2. Lepehne, Georg: Die klinische Bedeutung der Bilirubinbestimmung im menschlichen Blutserum, Deutsche med. Wchnschr. 49: 611, 1923.
3. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. 119: 480, 1937.
4. Vaughan, S. L., and Hubbard, R. S.: Van den Bergh Reaction of Bilirubin in Xanthochromic Spinal Fluid, Proc. Soc. Exper. Biol. & Med. 32: 618, 1935.
5. van den Bergh, Hijmans A. A.: Der Gallenfarbstoff im Blute, Leiden und Leipzig, 1918.
6. Aschoff, Ludwig: Ueber Bildungs- und Ausscheidungsstörungen der Gallenfähigen Substanzen (Dyscholie), besonders des Gallenfarbstoffs (Ikterus), Acta path. et microbiol. Scandinav. 5: 338, 1928.
7. McNee, J. W.: Gibt es einen hämolytischen Icterus? Med. Klin. No. 9, 1125, 1913. Experiments on Hemolytic Icterus, J. Path. & Bact. 18: 325, 1914.
8. Lepehne, Georg: Pathogenese des Icterus, Ergebn. d. inn. Med. u. Kinderh. 20: 221, 1921.
9. Eppinger, Hans: Hepato-lienale Erkrankungen, Berlin, 1920.

A SIMPLE METHOD FOR DETERMINING PLASMA DENSITY AND PLASMA PROTEIN CONCENTRATION*

Eric Ponder, M.D., D.Sc., Mineola, L. I.

In the course of an investigation on water balance and plasma protein concentration I have had occasion to make large numbers of plasma protein determinations by a modification of the Linderstrøm-Lang method for measuring density, which is so convenient and accurate that it is worth while drawing attention to it.

The only apparatus required is a glass cylinder of 100 to 200 ml. capacity, graduated in milliliters. At least every 10 ml. graduation mark should run all around the cylinder, so as to enable one to avoid parallax in making readings. The cylinder is placed at eye level on a well-lit shelf shielded from drafts, in a room in which rapid changes of temperature do not occur. It is half-filled with a brombenzene-kerosene mixture of density 1.0485, made by mixing 35 ml. of brombenzene and 65 ml. of kerosene (density of brombenzene = 1.497; density of kerosene = 0.807). On top of this is layered about 50 ml. of a lighter mixture of density 1.0210, made up of 31 ml. of brombenzene and 69 ml. of kerosene. A rod, with a ring which just fits inside the cylinder at right angles to it, is moved gently up and down about a dozen times in the region of the

*From the Nassau Hospital, Mineola.
Received for publication, August 25, 1942.

interface between the heavier and the lighter fluids, and in this way a remarkably even gradient of density is produced, usually between the 20 ml. and the the 80 ml. marks on the graduation of the cylinder.

A series of standards are prepared from the commercially supplied potassium sulfate standard of density 1.0551[1] by the addition of distilled water in proportions indicated in Table I. These standards are kept in dropping bottles with well ground-in pipettes. A drop of each standard is dropped into the fluid in the cylinder, through which it descends until it reaches a level with a density the same as its own. The drop is formed on the end of the pipette, dipping below the surface of the fluid; on withdrawing the pipette, the drop breaks off. Its size is not very important, but 5 to 15 c.mm. is a convenient size. In this way the variations in the density gradient in the cylinder are marked off by the position of the six drops of known density, which correspond to six readings on the scale on the cylinder. In making the readings, care must be taken to avoid parallax. If the densities of the drops are plotted against their position on the scale, a remarkably good straight line usually results, showing that the density gradient is linear. Even if it is not, the graph can be used for finding the density of the gradient at any level. If the gradient changes, as it may on account of variations in temperature, etc., the positions of the standard drops change with it, and they always mark the six positions of their own densities.

TABLE I

D = 1.0551 ML.	WATER ML.	D OF MIXTURE	CORRESPONDS TO PROTEIN, GM. PER 100 C.C.
18.0	12.0	1.0330	8.89
16.5	13.5	1.0303	7.96
15.0	15.0	1.0276	7.04
13.5	16.5	1.0249	6.11
12.0	18.0	1.0221	5.17
10.5	19.5	1.0193	4.23

If a drop of plasma from heparinized blood is allowed to fall through the fluid in the cylinder in the same way, it will stop at a position in the density gradient, which corresponds to its own density. A reading of this position on the scale and reference to the graph for the positions of the standard drops will give its density immediately, and its protein content can be found from the density in the usual way from tables, or from a graph prepared by plotting the last two columns of Table I. Since the lipoids of the drop and the brombenzene and kerosene are not mutually altogether insoluble, the drop tends to sink slowly from the position which it first assumes, and so its position should be read after a standard time, e.g., one minute from the moment it is dropped into the cylinder. For the same reason, small drops are better than large ones, and 5 to 10 c.mm. is a convenient size. I use medicine droppers drawn out into capillary pipettes for forming the drops.

When some experience has been gained, the method will be found to give values for plasma protein concentration correct to ±0.1 Gm. per 100 c.c. This is not as high a precision as is claimed for the falling-drop method, but both methods have the weakness that what they measure is plasma density, which depends on protein concentration only when the lipoid content is constant, and

so a precision sufficient to measure ±0.0003 in the figure for density is enough for all clinical purposes. The method is convenient in practice, and at least 20 determinations can be made before the fluids need replacement and a new gradient has to be formed. Unwanted drops can be removed by touching them with a small piece of wet filter paper attached to the end of a capillary pipette. The drops adhere to the paper and can be pulled up out of the brombenzene-kerosene mixture.

REFERENCE

Linderstrøm-Lang, K.: Distribution of Enzymes in Tissues and Cells, Harvey Lecture 34: 214, 1939.

Solutions of standard density used in the falling-drop method for determining density are supplied by Eimer and Amend, New York, and other scientific apparatus houses.

MEDICAL ILLUSTRATION

A SIMPLE TECHNIQUE FOR TIME LAPSE CINEMICROGRAPHY*

Adrianus Pijper, M.D., D.Sc., and Laurie B. Poole
Pretoria, South Africa

PURPOSE OF THE METHOD

New methods lead to new findings. Cinematography in many laboratories is still a novelty. Photographing the subject through a microscope at ordinary speed may show nothing beyond what the eye has noticed already, although even here the film often records something that has escaped detection by the eye alone. Slow motion cinemicrography analyzes movements too rapid to be observed by the human eye. In time lapse cinematography and cinemicrography we have methods that make visible what no human eye has the patience or the facility to observe.

Time lapse cinematography is the process of taking pictures at a slower rate than the one at which they are eventually projected. Movements too tedious or too slow to be watched can thus be condensed into processes which are easily followed on the screen. Audiences are familiar with the method as applied, for instance, to the unfolding of a flower. In time lapse cinemicrography the same method is used but the pictures are taken through a microscope.

New methods can only bring results in proportion to the frequency with which they are used. Time lapse cinemicrography so far has not been applied widely. The hesitation to put into regular use a method so full of possibilities is largely caused by technical considerations. We believe that the technical difficulties of the method are overestimated. Considerable work in this field has been done by Comandon. Splendid apparatus has been built by Rosenberger,[5] Harris,[2] and by Roger,[6] but we feel that excellent results can be obtained by much simpler methods. In this paper we wish to show that time lapse cinemicrography is within the reach of most biologic laboratories. Our apparatus has been built by the simplest possible means and can be set up on an ordinary laboratory bench.

SPEED CONTROL

The chief part of the speed control is an ebonite disk, measuring $6\frac{1}{2}$ inches in diameter (Figs. 1*A* and 2*A*), which revolves once a minute. It is driven by an ordinary electric clock motor (Fig. 2*B*), which is operated on 60 cycle alternating electric current. There are 60 small equidistant holes drilled through this disk on a circle near the periphery. Tapering steel pins can be pushed into these holes. As the disk rotates, these pins make electric contacts which activate the shutter between the lamp and microscope, and also drive the camera and a counter.

*From Dr. A. Pijper's private laboratory.
Received for publication, July 20, 1942.

It is obvious that the longest lapse of time available with this apparatus is one minute, and the shortest time is a second. In addition, one can work at any number of seconds in between these two values by changing the number of pins on the disk. Up to the present we have found this time control ample for our requirements.

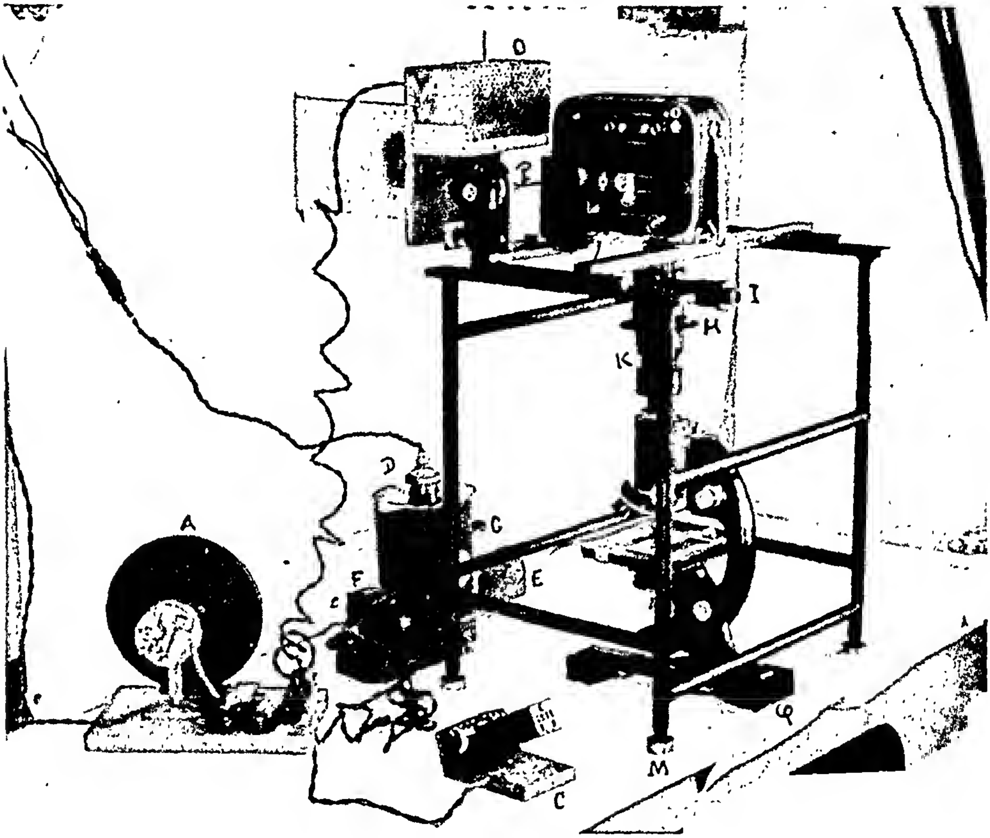


Fig. 1.—Side view of the apparatus.

A diagram of the wiring is given in Fig. 3. The steel pins make contact by depressing the metal tongues as they go through the lowest point of their rotation. The tongues are placed on top of one another and are arranged so that the one that activates the shutter between the lamp and microscope keeps this shutter open long enough for the tongue that works the camera to expose the film. The counter (Figs. 1*C* and 2*C*) is added in order to check the number of exposures made but is not an essential part of the apparatus.

SOURCE OF LIGHT

An Ediswan Pointolite lamp of 100 C. P. is used (Figs. 1*D* and 2*D*). A stronger lamp would be better but is not procurable under present conditions. An arc lamp, of course, is too unsteady for this kind of work. The shutter between the lamp and microscope (Figs. 1*E* and 2*E*) is made of light metal and is essential because it prevents exposure of the microbes to continuous light. It is lifted by a solenoid (Figs. 1*F* and 2*F*) and drops by its own weight. The shut-

ter can be kept raised permanently by pushing a lever (Fig. 1*G*) to the left. This is useful for making adjustments to the microscope.

THE MICROSCOPE

We use the large Zeiss stand FCG, as shown in Figs. 1 and 2, but any other heavy stand will serve. Since the dark field is the method of choice for this class of work, we use the Siedentopf cardioid condenser. To date we have worked mainly with fungal material, and a suitable combination of lenses was found in the Zeiss apochromat objective ×60 with iris and the Zeiss compensating eyepiece ×7. We have also used the Zeiss compensating eyepiece ×10. The distance between eyepiece and film is about 15 cm. These combinations resulted in magnification on the film of ×200 and ×310, respectively.

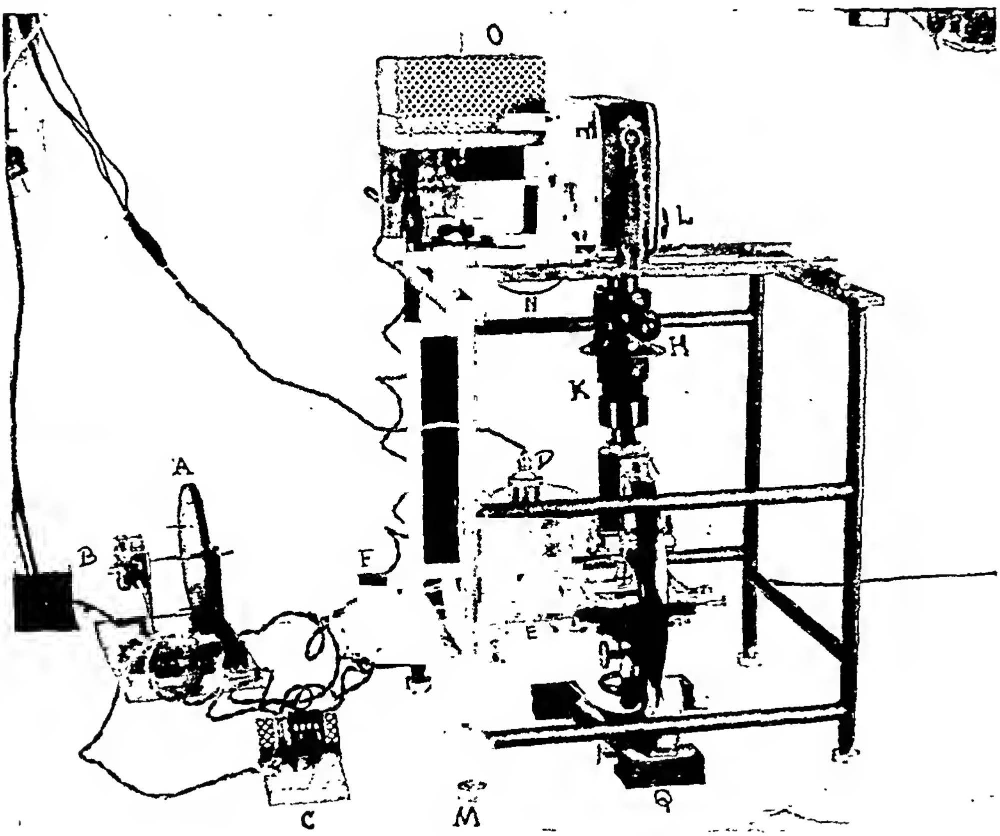


Fig. 2.—Front view of the apparatus.

THE PREPARATION ON THE SLIDE

For studying the growth of monilias, yeasts, and other fungi we place a small drop of a fairly thick suspension of spores on a microscope slide. A drop of melted Sabouraud agar cooled down to about 50° C. is added, and a cover slip is dropped on rapidly. Previous setting of the agar and moving of the cover slip must be avoided, since these lead to the formation of channels in the agar filled with thin fluid in which the spores swim about instead of lying still in solid agar. Petroleum jelly is applied around the cover slip. The microscope is then adjusted and the proper field is found in the usual way.

THE CAMERA

Our camera is a standard Movikon, with the lens removed and replaced by the beam splitter made by Zeiss for such purposes (Figs. 1*H* and 2*H*). Of course, any other make, or even a homemade one, will do. The one we use gives an additional magnification which is helpful in focusing. A suitable cap should be placed over the eye lens of the beam splitter (Fig. 1*I*), otherwise, as Barnard has pointed out,[1] extraneous light may reach the film. A lightproof connection between the microscope and camera is provided by a black velvet skirt (Figs. 1*K* and 2*K*) which fits into the sleeve around the eyepiece. The microscope and camera should not touch one another.

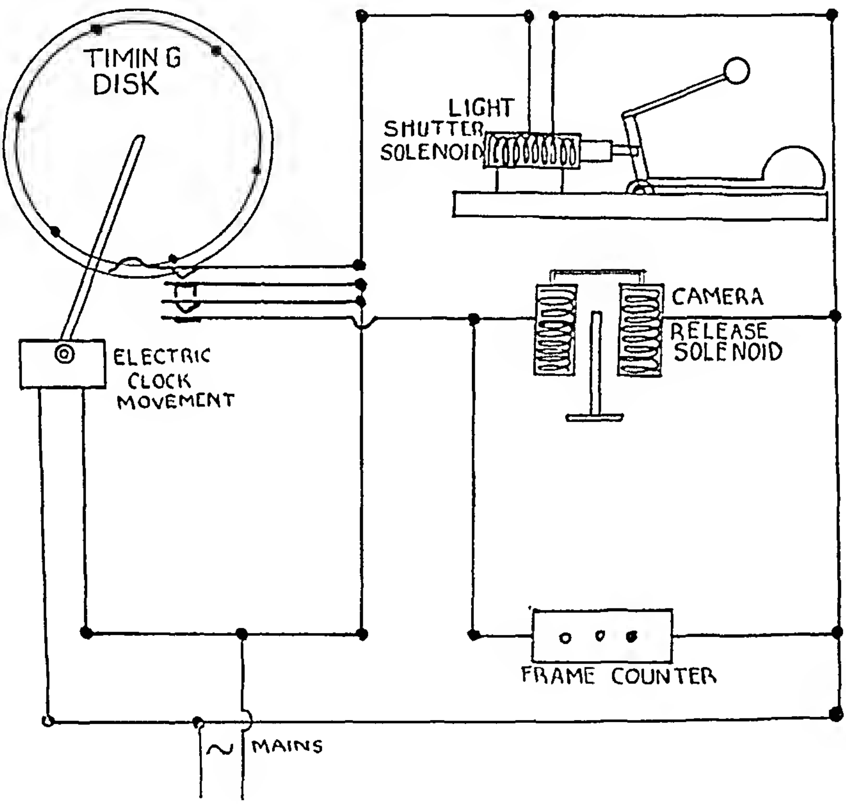


Fig. 3.—Wiring diagram of the apparatus.

The camera is supported on two horizontal flat iron bars, as seen in Figs. 1 and 2. It rests only on the front one and the posterior bar carries an upright piece of metal to which the camera is clamped by means of a clamping screw. This screw threads into the hole at the base of the camera provided for that purpose. The two horizontal flat bars also merely rest on the top of the iron stand which surrounds the microscope, as is shown in Figs. 1 and 2. From these illustrations it is seen that the camera can slide forward and backward with the two flat bars along the two round iron bars included purposely. This is necessary for accurate centering over the microscope. Slight movements to the side during centering are best executed by changing the position of the microscope. Centering is done through the beam splitter, but the beam splitter itself should be centered by looking at the picture on the film through the focusing magnifier which belongs to the camera (Fig. 2*L*).

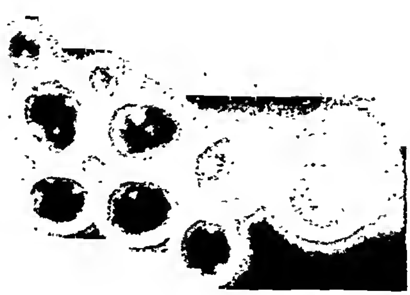
The stand around the microscope consists of four uprights, which are connected by sufficient crossbars to keep the structure rigid and to allow access to the microscope. The microscope can be handled easily with the stand in place. It can, however, be removed and be replaced again by means of the four brass cups (Figs. 1*M* and 2*M*) into which the conical ends of the four uprights fit tightly. These cups are screwed to the bench.

The Movikon camera possesses the essential single picture mechanism and is conveniently set in motion by means of a wire release (Fig. 2*N*). This wire release is activated through a solenoid (Figs. 1*O* and 2*O*), which is attached to a wall bracket and does not touch the stand or the camera.

Fig. 4.

Fig. 5.

Fig. 6.



Figs. 4, 5, and 6.—Frames from a film of budding Monilia cells.

The single picture mechanism of this camera does not allow an exposure longer than 1/25 second. This is ample for ordinary work but is insufficient for cinemicrography with a light source of 100 C.P., unless the lowest magnifications are used. In looking for a means to lengthen the exposure, we found that the Movikon camera has a shaft or rewinding spindle for making mixes. This shaft protrudes through the housing and makes a half revolution during each exposure. It was decided to slow down this shaft by attaching to it the dial

of an automatic telephone. The exposure was thus increased. Such a dial moves at a fixed speed which is controlled by a built-in governor. In Figs. 1 and 2 the dial is shown attached to an upright that is fixed to the posterior horizontal bar, to which the camera is attached in a similar manner. The dial center spindle is connected to the extended shaft of the camera by a universal coupling, a length of shafting, and a screw which is fitted onto the splined end of the coupling shaft. The splines and screw allow the dial to be disconnected quickly for winding the camera, but this cannot be done with the connection in place. By such an arrangement we found that the shutter action of the single picture mechanism was slowed down to ⅕ second.

The use of the camera spring for moving the film eliminates the geared electric motors which are usually deemed essential for time lapse cinematography. Our plan prevents their vibrations. The disadvantage of this method is that the spring must be wound at regular intervals. One winding suffices for 20 feet of film, and at a speed of one picture a minute each winding lasts ten hours. Frequent inspections of the field are necessary to make sure that nothing untoward is happening. Therefore, the occasional winding of the spring is of no great consequence.

VIBRATION

The danger of spoiling cinemicrographic pictures by vibration is often overrated. For speeds of 16 frames a second, for instance, or even 64 frames a second, we have shown previously that it is quite safe to place both the camera and microscope on the same iron optical bench,[3, 4] provided the eyepiece of the microscope does not touch the camera and the spring of the camera is used to move the film. For our time lapse cinemicrography with its longer exposures we found it safe to place the whole outfit on an ordinary wooden laboratory bench, if the precautions mentioned were observed. The weight of the camera with its attachments holds it steady. In addition, the camera with the posterior horizontal bar and the telephone dial can be lifted up and put aside as one piece by simply undoing the wire release from the camera. The anterior horizontal bar is loose and thus the microscope is ready for ordinary use at a moment's notice.

There is still another feature to explain. We prevent vibration of the microscope by placing its base on soft rubber pads (Figs. 1*Q* and 2*Q*). It is held in place by a long fastening screw which goes through a hole in the wooden bench and reaches the base of the microscope from underneath. The hole through the bench is lined with soft sponge rubber, and there is a rubber washer separating the head of the fastening screw from the bench beneath. This arrangement allows slight movements of the microscope from side to side and assists in centering.

FILMING OF BUDDING MONILIA CELLS

By means of the apparatus described we have made a film of budding Monilia cells, from which frames are reproduced in Figs. 4, 5, and 6. In the film the budding cell of Fig. 4 can be seen to produce by repeated budding and separating, first, the three cells of Fig. 5, and finally the seven cells in Fig. 6. It shows that before budding takes place there is great activity of the particles that can be seen in the cells. Usually this is near the part of the wall that is

going to break down. The yielding of the cell wall is a surprisingly sudden process compared with the following development of the bud. It is again accompanied by great activity of the particles, which can be seen making their way into the new cell. As a rule, it is possible to keep in focus the communicating opening between the two cells. The cell wall of a new cell is at first much thinner than later. As soon as it reaches the thickness of the old cell, the opening closes and often the cells separate with a sudden jerk.

SUMMARY

A technique is described which enables biologic laboratories possessing ordinary equipment and the services of a skilled technician to make time lapse cinemicrographic 16 mm. films. The apparatus is put together from simple materials, can be used on an ordinary wooden laboratory bench, and can be assembled and taken apart in a few moments, so that the microscope remains available for ordinary use.

REFERENCES

1. Barnard, J. E., and Welch, F. V.: Practical Photomicrography, London, 1925.
2. Harris, P.: Automatic Operation of Ciné-Camera and Lighting Arrangements for Time Lapse Studies, Nature 142: 834, 1938.
3. Pijper, A.: Microcinematography of the Motile Organs of Typhoid Bacilli, J. Biol. Photographic A. 8: 158, 1940.
4. Pijper, A.: Microcinematography of the Agglutination of Typhoid Bacilli, J. Bact. 42: 395, 1941.
5. Rosenberger, H.: Scientific Apparatus and Laboratory Methods: A Standard Microcinematographic Apparatus, Science 69: 672, 1929.
6. Roger, H.: A New Camera Timer for Time Lapse Cinematography, J. Soc. Mot. Pict. Eng. 32: 549, 1939.

THE MINIATURE CAMERA APPROACH TO VISUAL EDUCATION*

George Fiske Johnson, Ph.D., State College, Pa.

IT IS NOW almost six years since the miniature camera first attracted the serious attention of educators in the United States as a new approach to effective visual instruction. The growing enthusiasm among the professions for this type of camera and its products as an aid in teaching and in recording scientific data is revolutionizing the photographic and visual aid programs of many institutions.

The possibilities of the miniature camera and related equipment in photographing medical subjects, especially in color, have been reviewed previously in this JOURNAL. Jeter and Hull[1] pointed out several years ago the value of using 35 mm. color film in miniature cameras as a means for securing 2 by 2 inch slides for illustrative purposes. "The pathologist and the pathologic department of every hospital should assume teaching responsibilities," they wrote. "We believe color photographs, as carried out by the simple, inexpensive method described, is now practical and, furthermore, adds greatly to the facilities of teaching."

*From the Agricultural Extension Service of the Pennsylvania State College.
Received for publication, July 21, 1942.

Tomlinson[2] recently called the attention of the medical profession again to miniature cameras, 35 mm. color film, 2 by 2 inch slides, illuminators for displaying color transparencies, and especially their application to photomicrography.

DeLucchi,[3] reporting on a survey of 55 medical school hospitals, clinics, biologic photographic departments, and medical laboratories, writes: "There is a very noticeable increase in the use of the smaller 2 by 2 inch Kodachrome slides (photographed usually in miniature cameras). . . . A numerical checkup will show that the demand has been so great that the smaller-sized slide is definitely and rapidly becoming part of the routinely produced material in a majority of biological laboratories. The field of photography as used to illustrate surgical procedure has also been invaded by the 2 by 2 inch Kodachrome slide. More and more this small size slide is being used to augment 16 mm. Kodachrome motion pictures, and in one department has replaced the motion picture to a large extent. It is felt that a series of pictures taken during the process of an operation are excellent for teaching. The cost of such a series is less than a motion picture covering the same procedure. It is to be noted that three out of four new photographic laboratories (less than one-year old) are using 2 by 2 inch slides. . . ."

Other writers, especially in the field of biology, such as Brubaker and Holinger,[4] Waller and Breedis,[5] Footer,[6] and Koch,[7] have recognized this new trend in photography and visual education and have recorded many valuable ideas and experiences.

I have been associated with the development of a visual aid service in the division of agricultural and home economics extension at the Pennsylvania State College for the past seven years. This service employs 200 county farm and home economic representatives and state-wide subject matter specialists who conduct demonstrations on better farm and home economic practices and assist with 10,000 educational programs in rural communities annually.

Our enthusiasm for the miniature camera was generated in 1937 when it was demonstrated that a $15 camera (more expensive now) taking the new 35 mm. color film could produce reasonably true-to-color transparencies at a film cost of 15 cents each. We saw in this simple, inexpensive equipment the means of improving the photographic and visual aid program for our workers. The reasons for this were: (1) the equipment was light and easily carried; this was an extremely important advantage because our workers were traveling educators; (2) the equipment was less expensive than that available in the past; (3) the miniature camera loaded with color film was more foolproof and more exhilarating in results attained than larger cameras with black-and-white film; (4) the unit cost of the visual aids secured was substantially less than that prevailing with either the standard lantern slide or 16 mm. motion pictures. We now have in our organization 107 miniature (35 mm. film) cameras costing from $15 to $175 each.

Slides, 2 by 2 Inches, Lead as Visual Aids.—How revolutionary recent improvements in visual aids have been in our case can be cited specifically. The number of standard-sized black-and-white lantern slides in use decreased from 5,000 in 1935 to fewer than 500 (used infrequently) in 1942, and in their place have come almost 25,000 of the new 2 by 2 inch slides taken with miniature

cameras, mostly in natural color. We produced more than five times as many color slides in four years (1937 to 1941) as standard-sized black-and-white slides in twenty years (1917 to 1936). Even more significant was the fact that a complete equipment change requiring the adapting of old projectors or the purchase of new projectors was necessary to effect this change-over in the type of slide used.

Fig. 1.—County Agricultural Agent Russell M. Smith, Forest County, Pa., with a portion of the 800 2 by 2 inch slides which he has produced and utilized more than 300 times in the past four years on a total equipment and film expenditure of less than $225.00.

Screens for showing pictures changed in character completely during this seven-year period. Most screens in 1935 were white cloth on which color slides would not show up clearly in the daytime in rooms that could not be darkened completely. Only one solution could be found and that was to buy the beaded surface type of screen. A few demonstrations were extremely convincing, so that today we have 82 beaded surface screens, 77 in county offices and 5 in the State College office. Each county has at least one of these screens, and 11 counties have two each. The folding base tripod screen is most popular.

The trend in the use of visual aids since 1935 is shown in Fig. 3. The use of 2 by 2 inch slides has increased from zero in 1936 to almost 50 per cent of all meetings in 1942; 16 mm. movies (mostly silent), of which we made over 150 reels, gained from 5 to 15 per cent; large lantern slides and film strips decreased from 25 to 1 per cent; visual aids other than projected pictures dropped slightly from 30 to 25 per cent; and meetings with no visual aids decreased from 35 to 10 per cent. It will be observed that the 2 by 2 inch slides are used in a larger percentage of meetings today than all forms of projected pictures employed only seven years ago.

The mistake is made of regarding the 2 by 2 inch slides as nothing more than the large lantern slide in a new dress. This transparency is a *new visual medium*. Because of its low film and processing costs each step in a process

can be recorded economically. Furthermore, the mechanics of the camera are so simple that a sequence of pictures can be taken quickly. In this sense the miniature camera is a cross between the motion picture camera and the more cumbersome cut film or film pack reflex or view camera. Likewise, when skillfully used in the lecture room the resulting pictures retain all advantages of the slide medium while acquiring some of the advantages of the motion picture.

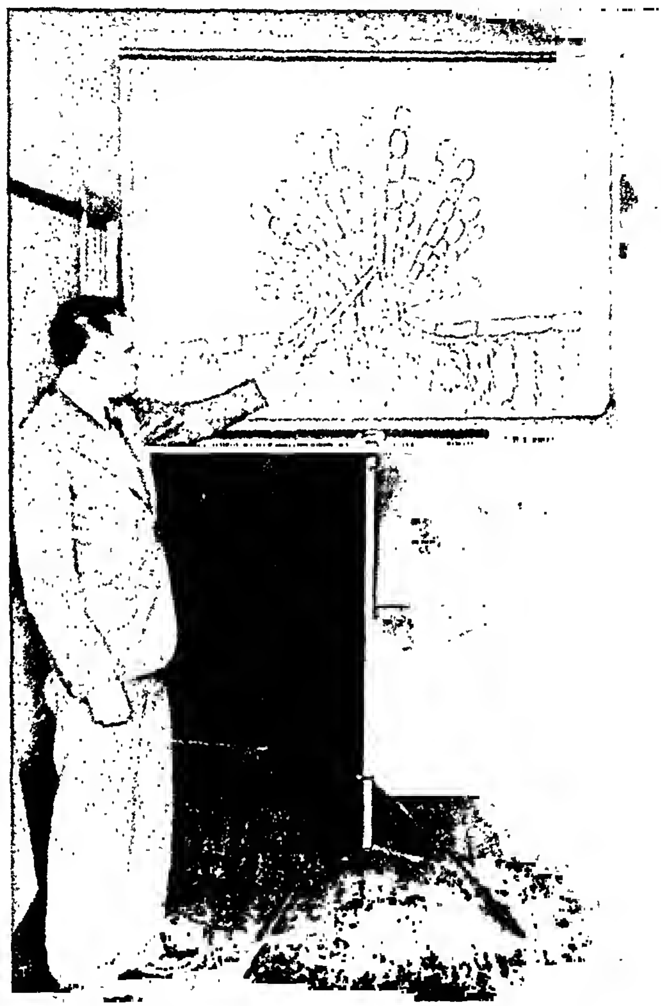


Fig. 2.—The folding base tripod beaded surface screen in use. Note that it can be elevated to shoulder height.

Advantages of Slides over Motion Pictures.—Although physical completeness is attained with a well-photographed motion picture of an operation or method demonstration, this does not mean that completeness of understanding is always reached, even when fully explained. The skillful lecturer can often take a well-photographed series of slides of this same operation or method demonstration and secure more complete and lasting understanding of the operation because: (1) the most critical stages or steps in the operation can be retained clearly on the screen for an indefinite period for detailed explanation;

(2) contrasts and comparisons can be made most easily and, if necessary, remade to fit the mental response of the audience; (3) in subsequent discussion any one scene can be selected quickly and returned to the screen; (4) the still transparencies can be placed in an illuminator and the entire sequence can be studied at one time. Of course, the ideal is a motion picture and a series of still transparencies of the same operation. This is often impractical, however, due to the cost or the impossibility of repeating the exact operation.

To accomplish the most with slides, we find that a 300 or higher wattage projector with forced draft or other means of thorough ventilation is necessary. This type of equipment enables a lecturer to retain a slide in the projector indefinitely without damage and still secure good projection with sufficient light in the room for note taking.

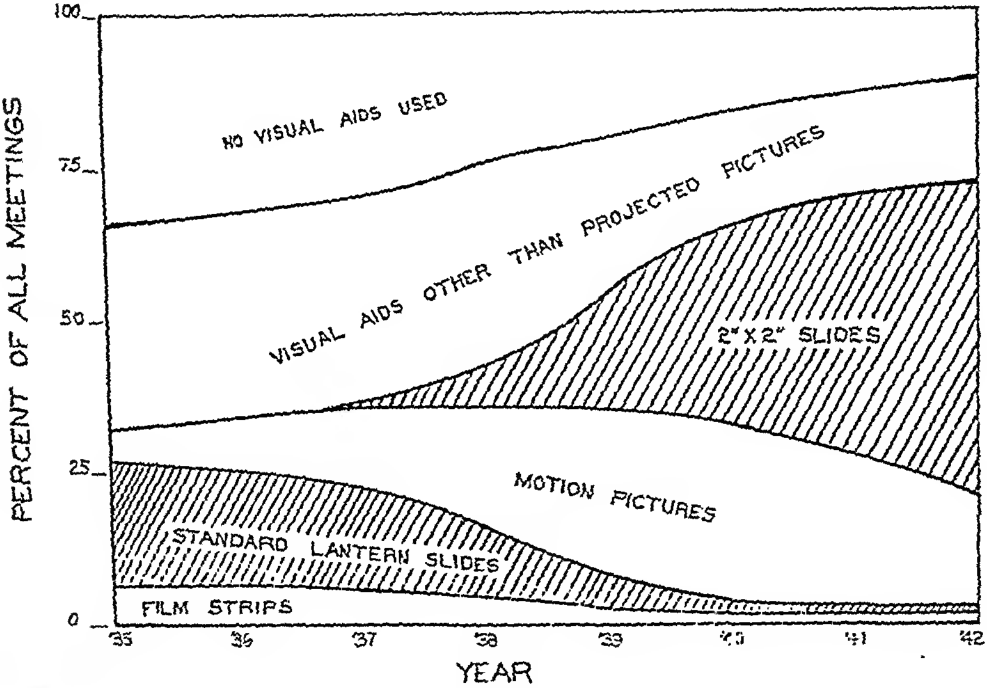


Fig. 3.—Trend in the use of visual aids in indoor subject matter meetings conducted by the Pennsylvania Agricultural Extension Service, 1935 to 1942.

One important factor in our plan is that assistance and encouragement are given for workers to produce their own visual aids. The development of the miniature camera, color film, and the exposure meter has been a great stimulus in this direction. The miniature camera, as we use it, is a lantern slide camera because color film is used, and when the shutter is clicked, a lantern slide is produced. This so simplifies the procedure of making lantern slides that over 80 of our workers are successfully producing these slides at the rate of 5,000 a year.

MINIATURE REFLEX CAMERA FOR PHOTOMICROGRAPHY

We find the 35 mm. film size reflex camera extremely practical for photomicrography. In this type of photography we employ a carbon arc light for illumination with either transmitted or reflected light, depending upon the

can be recorded economically. Furthermore, the mechanics of the camera are so simple that a sequence of pictures can be taken quickly. In this sense the miniature camera is a cross between the motion picture camera and the more cumbersome cut film or film pack reflex or view camera. Likewise, when skillfully used in the lecture room the resulting pictures retain all advantages of the slide medium while acquiring some of the advantages of the motion picture.

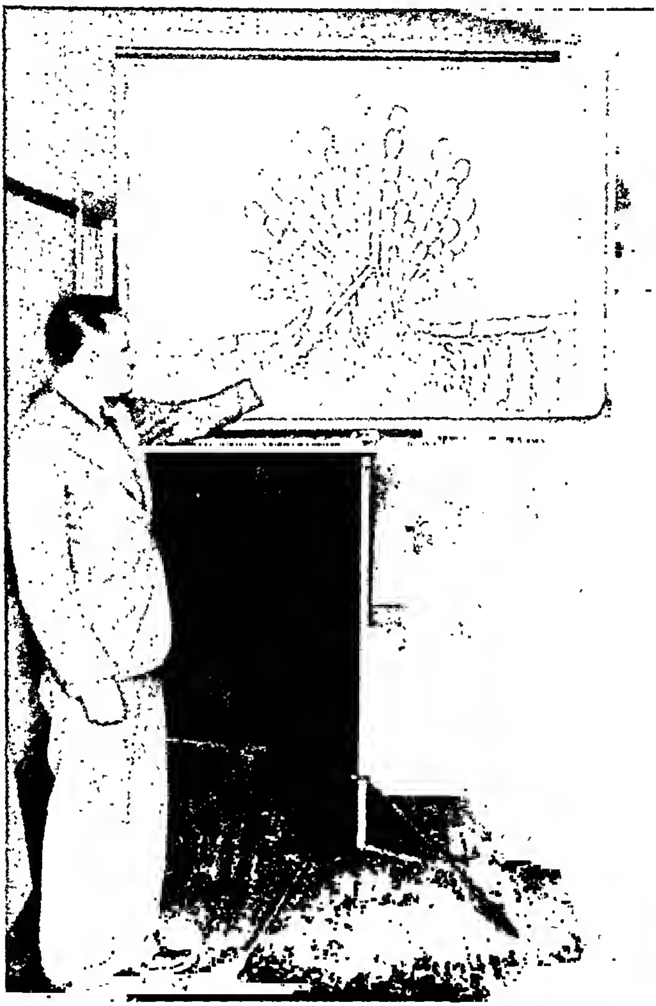


Fig. 2.—The folding base tripod beaded surface screen in use. Note that it can be elevated to shoulder height.

Advantages of Slides over Motion Pictures.—Although physical completeness is attained with a well-photographed motion picture of an operation or method demonstration, this does not mean that completeness of understanding is always reached, even when fully explained. The skillful lecturer can often take a well-photographed series of slides of this same operation or method demonstration and secure more complete and lasting understanding of the operation because: (1) the most critical stages or steps in the operation can be retained clearly on the screen for an indefinite period for detailed explanation;

(2) contrasts and comparisons can be made most easily and, if necessary, remade to fit the mental response of the audience; (3) in subsequent discussion any one scene can be selected quickly and returned to the screen; (4) the still transparencies can be placed in an illuminator and the entire sequence can be studied at one time. Of course, the ideal is a motion picture and a series of still transparencies of the same operation. This is often impractical, however, due to the cost or the impossibility of repeating the exact operation.

To accomplish the most with slides, we find that a 300 or higher wattage projector with forced draft or other means of thorough ventilation is necessary. This type of equipment enables a lecturer to retain a slide in the projector indefinitely without damage and still secure good projection with sufficient light in the room for note taking.

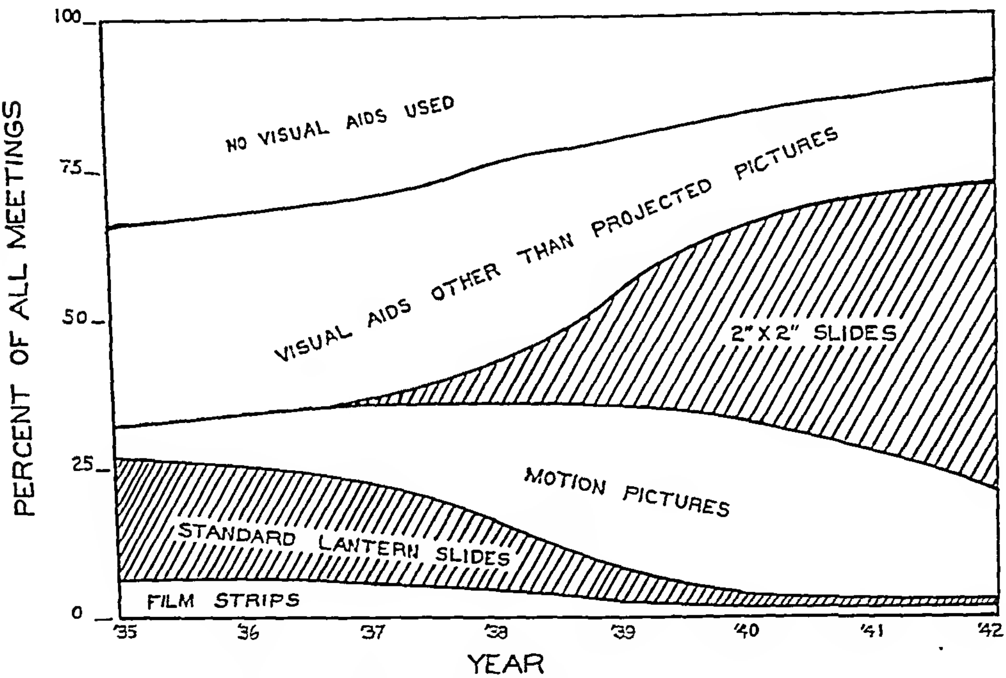


Fig. 3.—Trend in the use of visual aids in indoor subject matter meetings conducted by the Pennsylvania Agricultural Extension Service, 1935 to 1942.

One important factor in our plan is that assistance and encouragement are given for workers to produce their own visual aids. The development of the miniature camera, color film, and the exposure meter has been a great stimulus in this direction. The miniature camera, as we use it, is a lantern slide camera because color film is used, and when the shutter is clicked, a lantern slide is produced. This so simplifies the procedure of making lantern slides that over 80 of our workers are successfully producing these slides at the rate of 5,000 a year.

MINIATURE REFLEX CAMERA FOR PHOTOMICROGRAPHY

We find the 35 mm. film size reflex camera extremely practical for photomicrography. In this type of photography we employ a carbon arc light for illumination with either transmitted or reflected light, depending upon the

subject. The camera setup is as follows: The lens of the camera is replaced with a hinged microscope adapter which slips over the top of the microscope. The hinge enables the technician to swing the camera aside while searching for the proper field to be photographed. When this is determined, the camera is returned to the top of the microscope and focusing is completed through the

Fig. 4.

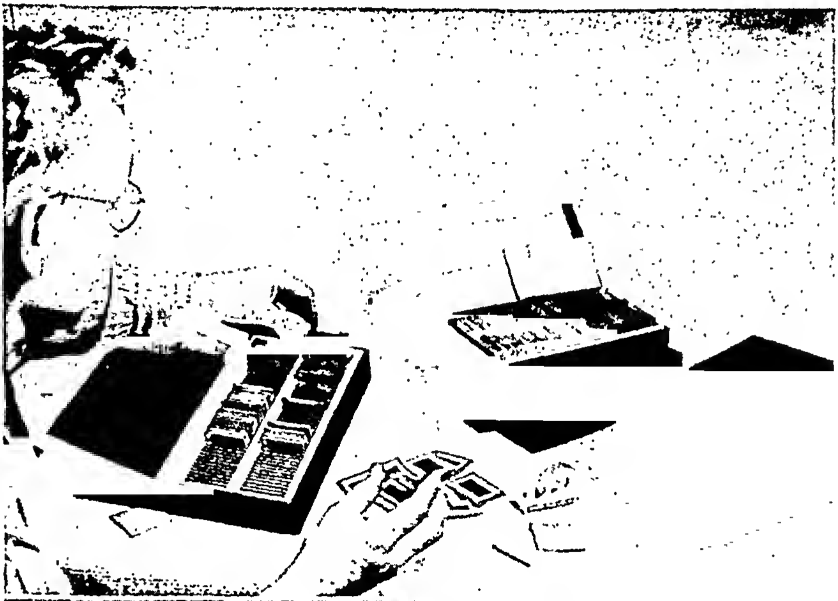


Fig. 5.

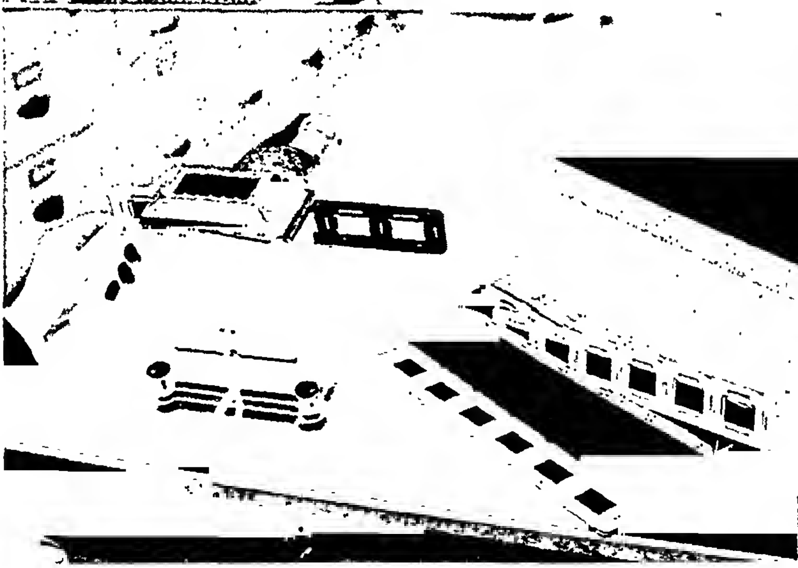


Fig. 4.—Filing 2 by 2 inch slides in 100 capacity boxes. Squares of cardboard with labels at the top are used for dividers.

Fig. 5.—The projector strip method of filing 2 by 2 inch slides.

reflex viewer. By using type A Kodachrome film and the carbon arc light, our experience indicates an exposure from one-tenth second to three seconds, based upon various light factors. We normally take three exposures, such as one-tenth, one-half, and one second, one-half, one, and two seconds, or one, two, and three seconds, depending upon our judgment of which light group fits the subject.

Fig. 6.

Fig. 7.

Fig. 6.—Sliding panels in a metal cabinet provide an efficient file for 2 by 2 inch slides.
Fig. 7.—Using the illuminator to arrange a series of slides for an illustrated talk.

The 2 by 2 inch Kodachrome transparencies can be duplicated successfully by the manufacturer at a cost of 25 cents each. The best duplicates are made from correctly or slightly underexposed views. Overexposures do not duplicate well. Color prints, measuring $2\frac{1}{4}$ by $3\frac{1}{4}$ inches, can be made from the transparencies by the manufacturer at a cost of 75 cents each. A 5 by $7\frac{1}{2}$ inch

size is made for $3.50. Fairly close-up subjects with distinct lines photographed on 35 mm. Kodachrome can often be converted successfully to a 5 by 7 inch black-and-white negative for a monochrome contact print of publication quality.

EFFICIENT FILES A NECESSITY

Since many of our departments and county offices have from 200 to 800 of the 2 by 2 inch slides, efficient filing has become essential to the best utilization of these vast visual aid resources. We have several types of files in use: (1) the box type with partitions for individual slides; (2) the panel type in which slides are filed in panels or cardboard holders and viewed by holding them before a lighted background; (3) the projector strip file in which slides are held in a cardboard projector strip that is used as a slide carrier in the projector (see Figs. 4 to 6).

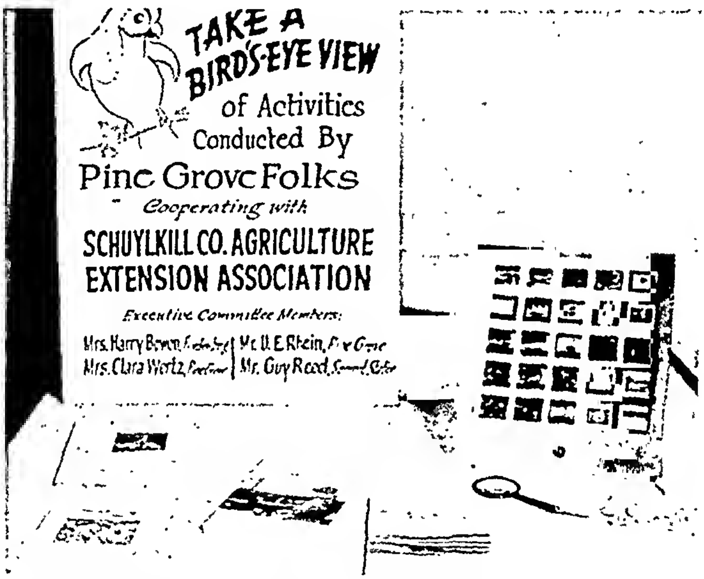


Fig. 8.—Inexpensive, simple and yet an extremely effective local community exhibit based upon the use of 2 by 2 inch slides in an illuminator. Note the magnifying glass on a string for use in looking at slides.

In general, a satisfactory file has the following characteristics: It is convenient to get slides into and out of the file with a minimum amount of time; it is not so complicated that great effort is required in keeping the file up to date; it is flexible in compartment spacing and new slides can be added without the continual handling of old slides. The object of an efficient slide filing system is to reduce slide handling and effort in filing to a minimum and at the same time make it possible to organize an illustrated talk quickly with all the material at hand. We have found that our workers who make the most of color slides in teaching are likely to accumulate at least 300 slides. To be most useful these must be in an efficient file. The cost of equipment for filing varies from 2 to 7 cents for each glass-covered slide, depending upon the system used.

THE ILLUMINATOR IN USE

In addition to a well-kept file of slides we find a relatively new piece of visual aid equipment called the *illuminator* useful in organizing a series of

these slides for lecture purposes. In simple terms, the illuminator is a ventilated boxlike structure with a white interior containing a 60 watt ordinary light bulb. The surface to be used for illuminating slides is of opal or ground glass. The slides are assembled on this surface.

With a 25 capacity slide illuminator available it is relatively easy to organize a series of slides for a lecture (Fig. 7). This piece of equipment eliminates the need of a preliminary showing of the slides on the screen, and it has the advantage of all slides being in view at one time. A magnifying glass can be used to enlarge the picture, if desired. Furthermore, we find the illuminator filled with color slides useful as the central feature of simple, inexpensive exhibits as shown in Fig. 8. In this we recognize the natural color slide as something more than a lantern slide to project onto a screen.

In our work we find a number of advantages in illuminated slides over projected pictures. The pictures can be viewed clearly in average indoor light without darkening the room. The need of carrying projection equipment and the difficulty of setup in small quarters filled with people are eliminated. Besides, a series of slides can be viewed and studied individually or as a sequence with all slides continually in view. On the other hand, the use of the illuminator is limited to situations where only a few persons look at the slides at one time, and it is impossible to show all types of views with equal effectiveness.

THE FUTURE

The 2 by 2 inch slide is definitely established as a visual aid medium of vast possibilities. As professional workers become more familiar with its many advantages the scope of its usefulness should continue to increase. It is likely to establish itself as an important supplement to the motion picture. For persons and institutions with limited budgets it will of necessity become the visual aid medium of primary consideration.

REFERENCES

1. Jeter, Hugh, and Hull, Wayne M.: Color Photography and Its Application in Medical Teaching, J. Lab. & Clin. Med. 23: 753, 1937-1938.
2. Tomlinson, Wray J.: Clinical Laboratory Photographs With a New Method of Preparing Photomicrographs, J. Lab. & Clin. Med. 26: 1064, 1941.
3. DeLucchi, Milton R.: 1941 Survey of Biological Photography, J. Biol. Photo. A. 10: 137, 1942.
4. Brubaker, J. D., and Holinger, Paul H.: The Larynx, Bronchi and Esophagus in Kodachrome, J. Biol. Photo. A. 10: 83, 1941.
5. Waller, R. K., and Breedis, C.: Photomicrography in Color, J. Biol. Photo. A. 10: 71, 1941.
6. Footer, Wilson: 35 mm. X-ray Slides, J. Biol. Photo. A. 10: 13, 1941.
7. Koch, Ferdinand L. P.: Color Photography of the Human Ocular Fundus, J. Biol. Photo. A. 9: 119, 1941.

DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TUBERCULOSIS, Pulmonary, The Weltmann Reaction, Owen, W. F., Jr., and Van Ordstrand, H. S. Cleveland Clin. Quart. 9: 135, 1942.

The Weltmann coagulation band was determined in 85 normal persons and a remarkably constant result was obtained, i.e., 6 to 6½.

In 75 cases of pulmonary tuberculosis, followed for a period of three to four months, an attempt was made to define how closely the coagulation band reflected the clinical picture and whether there was any correlation between the band and the sedimentation rate. In this group of 75 cases, 25 were chronic, arrested inactive cases, and in these the Weltmann reaction consistently showed a "shift to the right" and was a better indication of the clinical status than the sedimentation rate. In the remaining 50 cases the Weltmann coagulation band followed the clinical picture in 32 cases, or only 64 per cent, while the sedimentation rate was a good index in 40 cases, or 79 per cent. When the cases were divided into far advanced and minimal, it was shown that in the 27 far-advanced cases the Weltmann reaction was reliable in only 50 per cent as compared with 84 per cent with the sedimentation test. In the moderately advanced group of 20 cases, the sedimentation rate showed 60 per cent reliability and the coagulation band 80 per cent. There were only three minimal cases studied; the sedimentation rate was of no value in any while the Weltmann test was reliable in all.

Neither the Weltmann reaction nor the sedimentation rate is completely reliable or infallible in the diagnosis and prognosis of pulmonary tuberculosis. In these cases the sedimentation rate has been a somewhat better index of the clinical status of the patient. In doubtful cases it may be of value to do both tests.

In the chronic, arrested, inactive cases the Weltmann reaction consistently showed a "shift to the right" and was a better indication of the clinical status than the sedimentation rate.

There is no definite correlation between the Weltmann coagulation band the sedimentation rate.

PNEUMONIA, Capsular Polysaccharide in the Blood of Patients With Pneumococcic, Bukantz, S. C., deGara, P. F., and Bullowa, J. G. M. Arch. Int. Med. 69: 191, 1942.

Systematic determination of capsular polysaccharide, agglutinin, and precipitin titers were made on samples of blood of 135 patients with pneumonia caused by pneumococci of types I (32 cases), II (4 cases), III (40 cases), IV (1 case), V (9 cases), VII (33 cases), and VIII (16 cases) in a series rotated for treatment with sulfapyridine, with serum or with the two in combination.

Capsular polysaccharide was detected in the blood of 16 patients, or 11.8 per cent of the entire series. Eleven of the 16 had type III, 3 had type VII, and one had typo VIII pneumococcus pneumonia.

There was a significantly greater incidence of positive reactions for circulating capsular polysaccharide in the older age group, a tendency to greater incidence with increasing duration of disease, and an equal sex incidence.

The mortality for the patients in whose blood capsular polysaccharide was present was 62.5 per cent, while the mortality for all the patients with bacteriemia was only 29 per cent, and the mortality for the entire series was 11.8 per cent. The mortality for the patients without bacteriemia and without capsular polysaccharide in the blood was only 3 per cent.

Of the 10 patients whose serum gave a positive reaction for capsular polysaccharide and who were treated with sulfapyridine alone, 6 died. The illness of the 4 patients recovering

under sulfapyridine therapy alone ran a prolonged course, with a tendency to recurrence of fever, slow disappearance of capsular polysaccharide from the blood, and failure to produce antibody.

Of the 5 patients whose serum gave a positive reaction for capsular polysaccharide and who were treated with both sulfapyridine and serum, 3 died; two of these did not receive serum and sulfapyridine simultaneously, and the third received insufficient sulfapyridine. The 2 patients recovering after simultaneous serum and sulfapyridine therapy made much more satisfactory clinical responses. The sixteenth patient in whose blood capsular polysaccharide was detected died after treatment with serum alone.

CARTILAGE, A New Histochemical Reaction With High Specificity for Cartilage Cells, Hass, G. M. Arch. Path. 33: 174, 1942.

The fresh tissues, except when decalcification was necessary, was cut into sections with a freezing microtome set at 15 to 20 microns. The sections were divided into lots for preservation in water, alcohol, and solution of formaldehyde U.S.P. The sections in distilled water were subdivided into three groups. One group was used for immediate study. Sections of the second group were mounted on glass slides and dried rapidly in a vacuum desiccator. The third group of sections was placed in the refrigerator at 5° C.

It was impossible to obtain suitable sections of fresh bone, although osseous tissue often remained so attached to epiphyseal cartilages at the primary zones of ossification that a few observations could be made. Decalcification of formaldehyde-fixed or fresh tissue with 0.5 molar hydrochloric acid was necessary before good sections of bone were obtained.

Sections of the designated organs and tissues were exposed to the action of numerous combinations and concentrations of 0.5 molar hydrochloric acid, 0.5 molar sodium hydroxide, 0.20 molar sodium nitrite and 0.04 molar crystal violet. Optimum conditions for obtaining the desired reaction were as follows: A solution containing 10 c.c. of 0.5 molar hydrochloric acid, 4 c.c. of 0.04 molar crystal violet, and 1 c.c. of 0.20 molar sodium nitrite, or multiples thereof, was prepared. The reaction was allowed to proceed for forty-five minutes at room temperature. Then 0.5 molar sodium hydroxide was added slowly until a delicate fluorescence developed. Seven to 8 c.c. were required, the quantity being so adjusted that a fine precipitate formed in the reaction mixture about ten minutes after appearance of the fluorescence. After addition of the alkali, 20 c.c. of the reaction mixture was transferred to each of a desired number of 25 c.c. beakers. One section of tissue was placed in each beaker and kept there throughout the fifteen to twenty minutes required for the formation of a precipitate. After precipitation was complete, ether was poured on the surface of the solution. The sections were withdrawn from the reaction mixture through the ether layer, washed in distilled water, floated onto glass slides, blotted with filter paper, stained with aqueous hematoxylin, and mounted in glycerogelatin under a cover slip.

Similar results were obtained when the sections had been affixed to glass slides by preparatory desiccation in vacuo. When available in this form, the tissues were more easily handled than when they were floating in the solution.

This standard method was varied so that some data might be gathered with respect to the action of alcohol, acetone, ether, and 0.5 molar hydrochloric acid on the capacity of the tissue to react and on the stability of the reaction product once it had formed. By use of these reagents it was possible to make a microscopic study of cells prior to running the reaction, after a positive reaction had been obtained, and after eliminating all traces of the positive reaction. In other attempts to determine the nature of the reactive cytoplasmic component, the carmine stains for glycogen, the perosmic acid stain for lipids, the sudan IV stain for neutral glycerides, and the hematoxylin-eosin stains were prepared.

A positive reaction consisted of an aggregate of particles in the cytoplasmic area of a cell. The particles, which varied from 1 to 3 microns in diameter, were yellow, refractile, and discoid or spherical. They often had a biconcave form and always possessed curved margins.

REVIEWS

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

Gould's Medical Dictionary*

IF THERE is one book that every medical library and every physician *must* have, it is a medical dictionary. This new edition of Gould, long a favorite, will be found abreast of all the many and varied advances which have brought new and varied terms to medical literature. Numerous tables and new illustrations, many in color, make this new edition highly acceptable. It is highly recommended.

Textbook of Bacteriology†

THROUGH twelve editions Jordan's *Bacteriology* has established its own place. Dr. Burrows has taken the place of the late Dr. Jordan in editing the thirteenth edition. It has been somewhat enlarged and, with minor exceptions, completely rewritten. Chapters devoted to highly specialized fields, such as soil, industrial and dairy bacteriology, have been regrouped in a chapter on Bacterial Physiology. The relation of bacteria to disease is considered in its broad aspect. This applies especially in the section on virulence and resistance.

The editor has been conservative in his treatment of nomenclature and classification. He uses no single taxonomic scheme, although he favors most of the terms adopted in Bergey's *Manual*.

The new edition gives every promise of enjoying the popularity of its predecessors.

Foreign Bodies Left in the Abdomen‡

THIS is a summary review of the entire literature of the subject, analyzed and discussed with regard to methods of prevention and to legal responsibilities.

The first record of a sponge left in the abdomen was in 1895, when a sea sponge was discovered. Sea sponges evidently were used quite frequently in the early days. Very few appear in the literature after 1900, although one was found in 1916, which apparently had been introduced in the same year.

In 1901 a towel, measuring one foot by two feet, was left in an abdomen.

The interval between loss and recovery of sponges is sometimes very long. After the fifth year secondary operations caused by sponges are usually due to intestinal complications, although nearly half of them become encapsulated, cause little or no trouble, and are found only incidentally in other operations.

Methods of preventing loss are discussed in much detail. These include sponge counts, metal tracers, and the continuous sponge.

Most of the instruments left in the abdomen are forceps, hemostats, and needles, although one report from Germany described a pair of spectacles.

Drains are lost often enough to be important. Some foreign bodies in the abdominal cavity were originally swallowed.

The legal decisions reviewed in considerable detail will be of greatest interest to any who have been unfortunate enough to have left articles in the abdomen.

---

*Medical Dictionary. By George M. Gould, A.M., M.D. Revised by C. V. Brownlow. Flexible, ed. 5, 1,528 pages, illustrated. Plain, $7.00; thumb index, $7.50. The Blakiston Co., Philadelphia.

†Textbook of Bacteriology. By Edwin O. Jordan, Ph.D., late Andrew McLeish Distinguished Service Professor of Bacteriology, University of Chicago; and William Burrows, Ph.D., Assistant Professor of Bacteriology, University of Chicago. Cloth, ed. 13, revised, 731 pages. W. B. Saunders Company, Philadelphia and London, 1941.

‡Foreign Bodies Left in the Abdomen. The Surgical Problems—Cases, Treatment Prevention. The Legal Problems—Cases, Decisions, [illegible]. By Harry Sturgeon Crossen, M.D., School of Medicine, Washington University; [illegible] Crossen, *LL.B.*, School of Law, Washington University, St. Louis, Mo. [illegible] with 212 illustrations including 4 color plates. The C. V. Mosby Company, St. Louis, 1940.

Approved Laboratory Technic*

THE third edition of the *Approved Laboratory Technic* includes such new procedures as the quantitative determination of prothrombin, methods for semen examination, the determination of urobilinogen in the urine and feces, hippuric acid in the urine, serum lipase determination, the simplified Kolmer complement fixation test, the Eagle modification of the Wassermann, determination of vitamin C in urine and plasma, determination of sulfonamide compounds in the blood and urine, and determination of thiocyanates in the blood.

This book was originally prepared under the auspices of the American Society of Clinical Pathologists.

In addition to the authors, twenty-eight men have collaborated in the preparation of certain sections.

Science and Seizures†

A VERY readable popular book for the use of the patient with epilepsy or migraine, this work was written from the point of view of neuropsychiatry. The patient with epilepsy (Lennox prefers to call it seizures) will get a clearer understanding of his condition from perusal of this book and will be relieved of the feeling that he is stigmatized by his disease. For this purpose the book should be widely recommended.

Physicians, even neuropsychiatrists, will also enjoy it and, unless they have kept up with the most recent advances in the study of seizures, they will gain much from its perusal.

Dr. Lennox devotes considerable space to discussion of the importance and significance of electroencephalograms. Some workers in the field may feel that the generalizations concerning the significance of the electroencephalogram are somewhat premature, not as yet generally accepted. The deductions, however, are based upon fact as it is known today and certainly add to the interest of the volume.

Cardiac Classics‡

CARDIAC CLASSICS contains reprints and translations of fifty-two contributions by fifty-one men who have done most to advance our knowledge of cardiology. Aside from the fun that one derives from reading those essays which have made history in medicine, there is a utilitarian need for this type of volume for those who are writing today. Often quotations from earlier investigators are distorted successively by men who fail to go back to the original source for their quotations. The truth of this is so obvious to those who do much writing that examples need not be given. Often the misquotations actually change the entire sense of a remark.

Another realization came to the reviewer as he went through the pages of this excellent work: how few essays written by medical men deserve reprinting and rereading. How much print paper is wasted with rehash and unimportant stuff.

*Approved Laboratory Technic—Clinical Pathological, Bacteriological, Mycological, Parasitological, Serological, Biochemical and Histological. By John A. Kolmer, M.S., M.D., Dr. P.H., Sc.D., LL.D., L.H.D., F.A.C.P., Professor of Medicine, Temple University; Director of the Research Institute of Cutaneous Medicine, Philadelphia; formerly Professor of Pathology and Bacteriology, Graduate School of Medicine, University of Pennsylvania; and Fred Boerner, V.M.D., Assistant Professor of Bacteriology, School of Medicine and Graduate School of Medicine, University of Pennsylvania; Bacteriologist, Graduate Hospital, Philadelphia. Cloth, ed. 3, 921 pages, $8.00. D. Appleton-Century Company, New York and London, 1941.

†Science and Seizures: New Light on Epilepsy and Migraine. By William Gordon Lennox, M.D., Sc.D., Hon. Assistant Professor of Neurology, Harvard University Medical School; Visiting Neurologist, Boston City Hospital; President International League against Epilepsy; Vice President Laymen's League against Epilepsy; Secretary Harvard Epilepsy Commission. Cloth, 258 pages, with 10 illustrations, $2.00. Harper & Brothers, New York and London, 1941.

‡Cardiac Classics—A Collection of Classic Works on the Heart and Circulation With Comprehensive Biographic Accounts of the Authors. Fifty-Two Contributions by Fifty-One authors. By Fredrick A. Willius, M.D., M.S. in Med., Chief, Section of Cardiology, the Mayo Clinic; Professor of Medicine, the Mayo Foundation for Medical Education and Research, the Graduate School, the University of Minnesota; and Thomas E. Keys, A.B., M.A., Reference Librarian, the Mayo Clinic; formerly Carnegie Fellow, the Graduate Library School, the University of Chicago. Cloth, 858 pages, $10.00. The C. V. Mosby Company, St. Louis, 1941.

The work starts with William Harvey and ends with Herrick's description of coronary occlusion.

One is disappointed in not seeing an essay from the pen of Thomas Lewis, but it is interesting to observe that there are more index references to his name than to that of any other cardiologist outside the favored fifty-one.

Dynamics of Inflammation*

AN OUTSTANDING contribution to our knowledge of inflammation, this book differs from the older pathologic concept in the sense that it is dynamic rather than static. The author analyzes the physiology and pathological physiology of the migration of leucocytes from the capillaries and their activities in areas of infection and damage, and associated chemical changes as well as modifications in these activities brought about by immunity and allergy. This has been the field of Menkin's original investigations for many years. In addition he brings to the monograph a wealth of his own material.

Although the volume represents a tremendous amount of original investigations, Dr. Menkin's conclusions, his interpretations of the mechanism of inflammation, are such as are now widely accepted. The evidence presented in favor of acceptance of the new trophic substance which he has named leukotaxine is logical.

ITEM

Antipneumococcic Action of Cinchona Derivatives

Mellon Institute, Pittsburgh, Pa., is distributing gratis to all interested specialists who request them copies of a publication entitled "Structure and Antipneumococcic Activity in the Cinchona Series."

Erratum

The first paragraph on page 1458 of the August issue of the JOURNAL, in an article entitled "Studies on the Clinical Significance of the Serum Proteins" by Benjamin M. Kagan, M.D., Richmond, Va., should read:

"There are in existence several colorimetric methods which, while less difficult to use, do not approach the simplicity of usual routine tests, *and* some of which are subject to errors up to 10 per cent."

*Dynamics of Inflammation. An Inquiry Into the Mechanism of Infectious Processes. By Valy Menkin, Department of Pathology, Harvard University Medical School. Cloth, 244 pages, $4.50. The Macmillan Company, New York, 1940.

Charles Virgil Mosby
1876-1942

Founder of The Journal of Laboratory and Clinical Medicine

The Editors of

The Journal of Laboratory and Clinical Medicine

mourn the passing of its founder

Charles Virgil Mosby

who has contributed much to the progress of medicine in the building of a publishing medium second to none in its special field, thus providing facilities for interchange of ideas and perpetuation of accomplishments in the sciences

"Medicine and surgery would still be primitive crafts had it not been for a few dauntless souls who were willing to record faithfully, by the written word, the results of their findings."

"The sculptor needs the granite block out of which to carve the image that has taken form and substance in his brain. On its unyielding face he carves the creatures of his imagination. We are the sculptors that carve day by day our form and likeness upon the rocks of time. Overcome the difficulties, surmount the obstacles, break down the barriers, and you carve deep and lasting. You cut your image so deeply upon the hearts and into the souls of men that time itself will never efface your memory."

—*From the writings of C. V. Mosby*

The Journal of Laboratory and Clinical Medicine

VOL. 28 DECEMBER, 1942 No. 3

CLINICAL AND EXPERIMENTAL

EPINEPHRINE AND EPHEDRINE ANALOGUES AND THEIR CLINICAL ASSAY

WARREN T. VAUGHAN, M.D., ROLLIN M. PERKINS, M.D.,* AND VINCENT J. DERBES, M.D.,* RICHMOND, VA.

DISCOVERY of the chemical structure of epinephrine by Abel (1897) and its synthesis by Stolz (1904) and Dakin (1905) have been followed by the synthesis of many derivatives possessing pharmacologic actions resembling those of epinephrine.

The early synthesis of sympathomimetic amines by Barger and Dale, gave impetus to this study. They studied aliphatic amines with no attached benzene ring. Amines with less than four carbon atoms had little activity. Those with from four to six had some pharmacologic action. Addition of the benzene ring to a two-carbon chain, with two carbon atoms separating the ring from the nitrogen produced clear-cut sympathomimetic action (phenylethylamine, Table I). The introduction of hydroxyl groups at the para or meta positions within the ring resulted in more effective epinephrine-like action.

Epinephrine has hydroxyl groups in both meta and para positions. This arrangement in the ring constitutes the catechol nucleus. We may describe epinephrine as a compound formed by the union of the catechol nucleus with a two-carbon aliphatic amine in which hydroxyl is attached to the proximal carbon and a methyl radical to the nitrogen.

It was natural to assay those compounds which closely resembled epinephrine in terms of the latter as the standard. The most easily measured epinephrine effect is elevation of the blood pressure. In early pharmacologic studies, epinephrine derivatives were graded according to pressor activity and its duration, with pressor response to epinephrine designated as unity. Pressor action was found to be controlled in great measure by the hydroxyl groups in

*Upjohn Research Fellows.

Received for publication, May 1, 1942.

the benzene ring. Tyramine differs from phenylethylamine only in the presence of a para hydroxyl group. The pressor activity of the former is double that of the latter. The addition of another hydroxyl group again doubles the ability of the compound to increase blood pressure (Table I, *4*). It will be noted in the last column of Table I that, as pressor activity increases, its duration becomes shortened.

TABLE I

CHEMISTRY AND PHARMACOLOGY OF SOME ADRENALIN-LIKE COMPOUNDS	 CH—CH—NH					PRESSOR ACTIVITY (ADRENALIN-1)	PRESSOR DURATION (ADRENALIN-1)
1. Phenylethylamine (PE)	H	H	H	H	H	1/200-1/80	3-4
2. Tyramine	OH	H	H	H	H	1/100-1/20	2
3. p-methoxy-PE	OCH_3	H	H	H	H	1/300	4
4. Dihydroxy-PE	OH	OH	H	H	H	1/35	1
5. Epinine	OH	OH	H	H	CH_3	1/12	2
6. Arterenol	OH	OH	OH	H	H	3/2	2
7. Epinephrine	OH	OH	OH	H	CH_3	1	1
8. Neosynephrin	H	OH	OH	H	CH_3		
9. Paredrine	OH	H	H	CH_3	H		
10. Benzedrine (beta-phenylisopropylamine)	H	H	H	CH_3	H	1/300-1/100	5-10
11. Propadrine	H	H	OH	CH_3	H	1/300-1/60	7
12. Ephedrine	H	H	OH	CH_3	CH_3	1/300-1/100	7
13. Corbasil	OH	OH	OH	CH_3	H	1/4	2
	Para	Meta	Beta	Alpha			

The pharmacologic activity of epinephrine (at least as regards its pressor effect) appears dependent upon the catechol nucleus. The nucleus apparently accounts also for the short pressor duration since it is rapidly oxidized. Efforts have been made to counteract this rapid oxidation. Methylation of the hydroxyl radical increases stability but causes loss in pressor effectiveness (Table I, *3*).

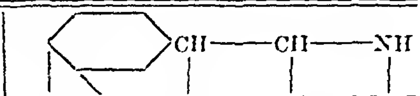
For better orientation we may review the prevailing hypotheses concerning the synthesis and destruction of epinephrine. There are gaps in the experimental evidence. The hypotheses cannot be considered proved, but for simplicity we shall present them didactically, because the theories are those most generally favored by chemists and pharmacologists and because they present a sequential picture, facilitating clinical discussion. Until there are better hypotheses, progress must be based on those now in favor.

Phenylalanine seems to be the precursor of epinephrine. It is an amino acid indispensable for growth. The possible succession of chemical changes brought about by enzymes is illustrated in Table II. The introduction of one hydroxyl in the benzene ring transforms phenylalanine into tyrosine, another amino acid. A second hydroxyl introduction produces dihydroxyphenylalanine. Next, the beta carbon is oxidized, after which the amino group is methylated. Decarboxylation of the alpha carbon produces epinephrine.

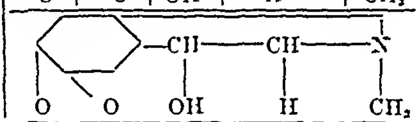
Destruction of epinephrine is an oxidative process, which can be brought about in two ways. In one, oxygen replaces the hydrogen of the hydroxyls in the catechol nucleus, producing an unstable quinone of epinephrine. Further internal oxidation reduction and molecular rearrangement result in an unstable indole derivative called adrenochrome, in which the nitrogen is attached to the benzene ring. Adrenochrome is inactive and appears responsible for the red brown discoloration of oxidized epinephrine.

This auto-oxidative process probably occurs in vitro. While it may occur in vivo, there appears to be a second process, incited by an enzyme, amine oxidase, which attacks many amines, converting them into aldehydes and ammonia (or in the case of epinephrine, into aldehydes and methylamine). Amine oxidase effect is comparable to that of cholinesterase against acetylcholine. Probably epinephrine is not destroyed too rapidly in the tissues because of the protective action of amino acids, which serve as hydrogen donors for epinephrine. Glutathione and ascorbic acid also serve as reducing substances. Both are abundant in the adrenal glands and probably participate in stabilization of epinephrine in the gland and in the blood leaving it.

TABLE II



SYNTHESIS AND OXIDATION OF EPINEPHRINE				CH	CH	NH
Synthesis	1. Phenylalanine	H	H	H	COOH	H
	2. Tyrosine	OH	H	H	COOH	H
	3. Dihydroxphenylalanine	OH	OH	H	COOH	H
	4. Oxidation of beta carbon	OH	OH	OH	COOH	H
	5. Methylation of amino group	OH	OH	OH	COOH	CH_3
	6. Decarboxylation	OH	OH	OH	H	CH_3
Oxidation	7. Quinone of epinephrine	O	O	OH	H	CH_3
	8. Adrenochrome	O	O	OH	H	CH_3



Briefly summarizing, the pressor activity of epinephrine and probably its other sympathomimetic effects are due primarily to the presence of hydroxyl groups in the benzene ring. This catechol nucleus also accounts for the evanescence of the response, epinephrine being rapidly oxidized by amine oxidase. The effectiveness of related synthetic compounds is also due to hydroxyl groups in the benzene ring.

Impetus was given the study of other synthetic compounds of potential clinical value by the identification of ephedrine (Table I). Ephedrine differs from epinephrine in the absence of hydroxyl groups in the benzene ring and the presence of a third carbon atom in the aliphatic chain in the form of a methyl group. Ephedrine effect is similar to that of epinephrine, but it does differ from it. Without the oxidizable catechol nucleus, ephedrine is not as easily broken down and is, therefore, effective when taken by mouth. For the same reason, its pressor activity is less, but the duration of pressor action is lengthened. Other physiologic effects, such as relief of bronchospasm, are similarly less pronounced.

Today there are over 200 compounds related to epinephrine and ephedrine which might have some sympathomimetic action. Of those related to ephedrine we shall use as illustrations only the four indicated in Table I. These illustrate the outstanding differences between the epinephrine group and the ephedrine group, summarized as follows:

In the absence of the catechol nucleus, the compounds are not destroyed as quickly.

The methyl group in the alpha carbon position prolongs pressor action but diminishes its intensity.

This methyl group may be responsible for the central nervous stimulation.

The addition of a phenolic hydroxyl group reduces the central stimulation. Paredrine has an arrangement in the benzene ring similar to that of tyramine, while its side chain is similar to benzedrine. It does not stimulate the nervous system. Corbasil is nonstimulating.

Ephedrine and related compounds, unlike epinephrine, produce tachyphylaxis. Successive doses given at short intervals exert progressively diminishing effect.

Gaddum's theory of the action of ephedrine, propadrine, benzedrine, and related compounds is an attractive hypothesis. Amine oxidase destroys epinephrine by oxidation. Ephedrine, like epinephrine, has an attraction toward amine oxidase. Indeed, its attraction is greater. Ephedrine combines with amine oxidase, thereby protecting the epinephrine normally present in the body. Consequently, epinephrine appears to become more active. Ephedrine potentiates epinephrine by blocking amine oxidase. Ephedrine being slowly oxidized, persists in combination with amine oxidase.

This would explain why small doses of ephedrine appear to increase the activity of epinephrine. Without having made quantitative studies, we have recognized the apparent increased effectiveness of an ephedrine-epinephrine combination as compared with straight epinephrine in clinical work and have used such a combination routinely for the past ten years. Our preparation consists of equal parts of 1:1,000 epinephrine solution and 3 per cent aqueous ephedrine. The dose of the combination has been the same as the dose of 1:1,000 epinephrine alone, usually 0.3 c.c. of the mixture.

Large doses of ephedrine sometimes appear to do the reverse, blocking epinephrine effect. The explanatory theory is that ephedrine, like epinephrine, may combine with the chemicals of the sympathetic myoneural junction. Ephedrine exerts no sympathomimetic action itself, and in this chemical combination it blocks epinephrine, obstructing the activity of the latter. With our small clinical doses this has not been a problem.

A suggested explanation of the central stimulation of benzedrine (Mann and Quastel, 1940) is based on the theory that these compounds combine with amine oxidase, blocking its activity. As stated above, the oxidation of certain amines releases aldehydes. Aldehydes exert an inhibiting effect on cerebral respiration (oxidation). Benzedrine binds amine oxidase so that it cannot oxidize the amines. Toxic aldehydes are not liberated. As a consequence cerebral respiration is less impeded.

CLINICAL APPLICATION

From time to time new synthetics of this series are proposed for clinical use. The first question usually is, "Is it as good as epinephrine or ephedrine?" The tendency is to weigh the effectiveness of any other compound in terms of these two. Propadrine, benzedrine, and neosynephrin are compared with ephedrine. Proprietary nebulizers with solutions having special trade names such as Vaponefrin or Neosol are presented, sometimes with the innuendo that the solution is superior to epinephrine. On questioning, one is usually informed

that the material is alpha methylamino-beta-hydroxyl dihydroxybenzene. Study of Table I indicates that this is epinephrine. For general sympathomimetic effect, nothing has as yet been found superior to epinephrine.

We do not imply that no such compound may eventually be found. Gunn has formulated four criteria for the ideal sympathomimetic: (1) it should be effective when given orally; (2) its action should be more prolonged than that of epinephrine; (3) it should be more certain than ephedrine; (4) there should be no central stimulation. The nearest approach in our past experience has been the mixture of epinephrine and ephedrine, which, however, still requires hypodermic administration.

Though the ideal sympathomimetic is not yet at hand, it may be that one compound is superior for one type of effect, while others are better for other actions. This is true with propadrine, benzedrine, neosynephrin, and paredrine. The question should not be "Are they as good as epinephrine or ephedrine," but rather, "Do they excel these in one or another activity, and do they have any superiority in lessened undesirable side effects?"

Epinephrine, for example, is effective in shrinking turgescent nasal mucosa. In fact, it is too effective, producing so much vasoconstriction with tissue anoxia that secondary vasodilatation may ensue, with consequent increased edema. One to 3 per cent ephedrine produces local decongestion, usually adequate, and not enough to cause anoxia. Neosynephrin appears to have special value here in that it is effective in much lower concentration (0.25 per cent). Although benzedrine produces undesirable central stimulation, its base is volatile. This gives it definite value as an inhalant in nasal allergy.

On the other hand, neosynephrin exerts little action when taken internally, except in large doses. It is about as effective as ephedrine in relaxing bronchospasm, but for adequate dosage it must be given hypodermically and is therefore inferior to ephedrine, although it has a slight superiority in that it exerts less nervous stimulation. Although benzedrine dilates the bronchi, this effect is not marked, and there is little change in respiratory rate, minute volume, or vital capacity. There is no unanimity of opinion concerning its action on gastrointestinal musculature.

We see therefore that neosynephrin and benzedrine base are superior compounds for topical application but possess no outstanding superiority with regard to other effects in the allergic diseases. The value of benzedrine in narcolepsy might be mentioned parenthetically, although this is not in the category of allergic diseases.

Paredrine exerts little action on the bronchi or gastrointestinal tract. It has pronounced pressor action and is not stimulating to the nervous system. Its outstanding effectiveness is as a mydriatic.

Propadrine exerts less central stimulation than ephedrine or benzedrine, has about the same pressor activity as ephedrine, and is about as effective when applied to the nasal mucosa. It may be given by mouth in the same dose or double the dose of ephedrine. Propadrine therefore finds use with patients who find ephedrine too stimulating.

Many other compounds have been studied, including kephrine, synephrin, arterenol, paredrinol, sympatol and epinine. Since none have superior effectiveness in relieving local or general allergic reactions, we shall not discuss them.

The list of compounds which might be superior in one or another respect is not complete, and the desirability of continuing the search is obvious, even though one must realize that a majority of the related compounds may be less effective than those now available.

PLAN OF INVESTIGATION

On the assumption that certain compounds may show superiority in relieving one or another but not necessarily all allergic responses, clinical investigation should include assay of sympathomimetic and allied responses in all tissues that lend themselves to objective investigation. Our preliminary program was outlined as follows:

Increased Capillary Permeability. A compound which will diminish capillary hyperpermeability, basically characteristic of the allergic response, should be of value, provided no undesired side effects accompany this action. Objective study might include the action of drugs upon experimentally induced histamine and allergen wheals. Epinephrine or ephedrine, or both, should be used as standards for comparison.

Localized subcutaneous edema of the angioneurotic type might be studied in its response to sympathomimetic drugs by determination of tissue tension in the edematous area (Vaughan and Pipes, 1940).

Both methods were employed, but it was found that tissue tension changes in wheals were not always sufficiently pronounced for reliable evaluation, and that the problem of producing angioneurotic edema at will in predisposed persons could not be easily solved. Tissue tension studies were therefore discontinued.

The action of compounds on whealing was studied in both normal and allergic persons.

Bronchial Obstruction. The obstructive phenomenon in bronchial asthma is probably due to local submucosal edema as well as bronchospasm. In the present study it is unimportant which is the dominant factor, since the desired therapeutic result, bronchial dilatation, would hold in either case.

Alexander and Kountz and others have shown that epinephrine and ephedrine increase vital capacity in asthmatic and in normal persons. Starr, also Richards, Barach and Cromwell suggest that maximum ventilation in liters per minute may be a better index of improved pulmonary function than vital capacity.

At the beginning, vital capacity studies were made. Later these were supplemented with ventilation capacity determinations.

Local Vasomotor Response. Here again it is unimportant whether the reaction in the nose is due to increased capillary permeability or vascular dilatation, or both. The therapeutic criterion is increased ventilation through the nose.

We have found the most satisfactory measure of nasal mucosal shrinkage in the Glatzel-Mulinos nasal mirror. This gives roughly quantitative results. Our adaptation of this method has been described elsewhere (Vaughan and Derbes, 1941).

Gastrointestinal Hypertonicity. There is no generally accepted objective method for clinically determining the response of smooth muscle in the intes-

tines. The methods used have yielded conflicting results (e.g., in the case of benzedrine). It seemed fruitless to repeat these procedures. Subjective tests were made with some persons subject to repeated gastrointestinal allergic reactions, manifested especially by distention. These patients tried different preparations repeatedly, not knowing which were being given, and expressed their conclusions as to which were more effective. We found propadrine and ephedrine most effective. Propadrine was preferred since it produced less jitters.

Toxic Allergic Fatigue. In our experience a phase of pronounced fatigue is an occasional accompaniment of food allergy, especially if the allergenic food is not rapidly removed by vomiting or diarrhea. This fatigue may appear within from thirty minutes to several hours, and usually lasts for twenty-four hours or longer. Certain persons appear to be especially susceptible to this response.

Here, again, any evaluation must be subjective. Two persons who were subject to allergic fatigue and were well acquainted with the manner of their response to allergenic foods evaluated different compounds in terms of comparable relief from 5 mg. of benzedrine. Benzedrine was most effective and had least unpleasant side effect in 5 mg. dosage. Propadrine hydrochloride, 25 mg., and neosynephrin hydrochloride, 10 mg. capsules, were somewhat less effective but had a definite benzedrine-like stimulating action and no unpleasant side effects. Beta-o-methoxyphenyl-n-propyl*di*methylamine hydrochloride,* 50 mg. capsules, was slightly lower in stimulating action than propadrine. Ephedrine caused little stimulation and much jitters.

It is regrettable that no adequate objective measures of therapeutic benzedrine effect are available. This drug in usual dosage produces no consistent or diagnostic change in metabolic rate, blood sugar level, or deep reflexes, or in the formed elements of the blood. Evaluation must be made in terms of elevation of mood, euphoria, lessened fatigue, enhanced motor and speech activity, feeling of increased efficiency and increased ability to concentrate, irritability and sleeplessness.

Undesired Effects. Notations were made of undesired responses, when present, such as palpitation, tremor, "jitteriness," blood pressure elevations, tachycardia, urinary retention, etc.

ORIENTATION STUDIES

A preliminary survey was made to determine whether the contemplated methods would be sufficiently sensitive. This evaluation included study of the blood pressure, pulse rate, and vital capacity and notation of the presence or absence of palpitation or jitters. Increase in the number of leucocytes and erythrocytes per cubic millimeter of blood was also determined (Table III).

We started with methyl racephedrine and beta-o-methoxyphenyl-n-propyl*di*methylamine hydrochloride† (Table IV). These were compared with epinephrine and ephedrine.

*This will hereafter be simplified to read, "methoxyphenyl-propyl*di*methylamine." The italicized -di- serves to differentiate this compound from a similar mono- compound which will be mentioned subsequently in this report.

†The analogues studied were prepared according to the methods described by Woodruff, Lambooy, and Burt.

Three tenths cubic centimeter of 1:1,000 epinephrine subcutaneously produced an average increase in blood pressure of 24 mm., an increase in pulse rate of 11, and an average increase in vital capacity of 272 c.c. This was usually accompanied by palpitation and jitters.

Ephedrine, 25 mg. subcutaneously, caused an average rise of 12 mm. blood pressure, an increase in pulse rate of 5, and an increased vital capacity of 167 c.c. This also usually caused jitters.

TABLE III

RESPONSE TO SUBCUTANEOUS EPINEPHRINE AND ANALOGUES	BLOOD PRESSURE INCREASE	PULSE RATE INCREASE	LEUCOCYTE INCREASE	ERYTHROCYTE INCREASE	AVERAGE INCREASE IN VITAL CAPACITY	PALPITATION AND JITTERS
Epinephrine 1:1,000 0.3 c.c.	24	11	2,600	150 M	272	Yes
Ephedrine 25 mg.	12	5	700	300 M	167	Yes
Methyl racephedrine 25 mg.	0	0	None	200 M	74	No
Beta-o-methoxyphenyl-n-propyldimethylamine hydrochloride 25 mg.	0	0	None	0	74	No

Methyl racephedrine and methoxyphenyl-propyl*di*methylamine caused no increase in blood pressure or pulse rate and no jitters. They did produce an increased vital capacity, less, however, than that produced by ephedrine (Table III). The second preparation caused a slight decrease in blood pressure.

These studies were made without knowledge of the previous laboratory results with animals. They were then compared. The correspondence between laboratory and clinical results appeared to be satisfactory. The pressor activity of ephedrine had been found to be from 1/100th to 1/250th that of epinephrine, while methyl racephedrine was 1/4,000th and methoxyphenyl-propyl*di*methylamine had been slightly depressor in animals. The correlation in bronchial relaxation was not as good. We found an average increase in vital capacity after methyl racephedrine of only 74 c.c.; and after methoxyphenyl-propyl*di*methylamine, 74 c.c. Isolated lung tests had indicated that the two new compounds, especially the latter, might be superior to ephedrine in relaxing bronchoconstriction. We had given them in the same dosage as ephedrine (25 mg. subcutaneously).

The preliminary studies indicate that in identical dosage neither of the substitutes is as effective as ephedrine in increasing vital capacity. Further, none of the three is as effective as epinephrine. However, two possibilities were obvious. First, the new drugs might be as effective as ephedrine, or more so, if given in larger doses. Toxicity studies indicate that much larger dosage may be safely employed (Table IV). If in larger quantities they do not produce jitters as does ephedrine, they might have value in therapy. The second possibility was based on the absence of pressor response and tachycardia. Some effective drug may be found which in adequate dosage causes no increase in blood pressure.

Five subjects were used in each vital capacity series, four normals and one asthmatic. Ephedrine produced increased vital capacity in all subjects.

Epinephrine failed to produce increase in one subject. and the two ephedrine analogues failed in two and three subjects, respectively.

The one asthmatic in the series had an increase of 400 c.c. after epinephrine. 70 c.c. after ephedrine, and none after methyl racephedrine or methoxyphenyl-propyl*di*methylamine.

The second phase of the orientation study involves disappearance of histamine wheals after medication. Wheals were produced by the injection of 0.03 c.c. of 1:1,000 histamine hydrochloride. They were fully evolved after five minutes. We first determined the time required for involution of this wheal. Thereafter, the drugs were injected subcutaneously five minutes after the intracutaneous injection of histamine.

No more rapid involution was caused by 47.1 mg. of racephedrine than in the controls, while 94.2 mg. of racephedrine caused as rapid involution as after 0.3 c.c. of 1:1,000 epinephrine. Subjective symptoms were as pronounced after 94 mg. of racephedrine as after epinephrine. Palpitation and jitters persisted for four hours. At the end of four hours another histamine wheal was produced. This wheal was smaller than the original, indicating an inhibiting effect from 94.2 mg. of racephedrine at the end of four hours.

We next compared the involution after 50 mg. of ephedrine with that after 46.4 mg. of methyl racephedrine and 45.8 mg. of methoxyphenyl-propyl*di*methylamine. Methyl racephedrine was less effective than ephedrine. The second product, in slightly lower milligram dosage, was comparable to ephedrine.

This observation, if substantiated by a larger series, might suggest a specific function of methoxyphenyl-propyl*di*methylamine in the control of allergic diseases associated with whealing or increased capillary permeability, especially since in comparable dosage (46 mg. vs. 50 mg.) less side effect (palpitation and jitters) was noted than after ephedrine.

The final phase of the orientation study consisted in therapeutic trial, giving the two preparations in capsule form to persons with active allergic symptoms.

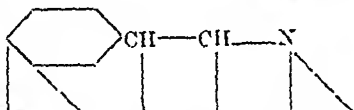
Fifty-two patients with various allergic symptoms were given methyl racephedrine, 51 patients received methoxyphenyl-propyl*di*methylamine (each in 25 mg. dosage by mouth). Of these, 34 were given both. Not all reported results. Thirty-two reported on the former, 35 on the latter, and 22 on both.

Of the 32 receiving methyl racephedrine, 19 reported subjective improvement and 2 described unpleasant symptoms. Of the 35 receiving methoxyphenyl-propyl*di*methylamine, 25 were helped and 2 reported unpleasant symptoms.

Eleven reported equal relief from both drugs, seven had no relief from either, three concluded that methyl racephedrine was more effective. five that methoxyphenyl-propyl*di*methylamine was more effective, and 14 concluded that the drugs were equally beneficial (Table V). The drugs were equally efficacious in asthma and rhinitis. Methoxyphenyl-propyl*di*methylamine was possibly more effective in urticaria.

These patient experiences appeared to confirm the objective studies in indicating that the drugs had some effectiveness in relieving bronchospasm and that the second was more effective in the relief of whealing.

TABLE IV

CHEMISTRY OF COMPOUNDS STUDIED							ACUTE TOXICITIES (MG. PER KG.)				CHRONIC TOXICITIES (MG. PER KG.)			
											ORAL IN RATS		ORAL IN RABBITS	
							INTRAVENOUS IN RATS	INTRAVENOUS IN RABBITS	ORAL IN RATS	ORAL IN RABBITS	DOSE	MORTALITY	DOSE	MORTALITY
Ephedrine	H	H	OH	CH_3	CH_3	H	110	70	400	1,100	250 100	50% 0	100	0
Methyl racephedrine	H	H	OH	CH_3	CH_3	CH_3	90			1,100	250	0	100	0
Beta-o-methoxyphenyl-n-propyldimethylamine hydrochloride	H	OCH_3	CH_3	H	CH_3	CH_3	40			500	250	0	100	0

At the termination of these orientation studies, we believed that we had evolved a reliable technique for clinical pharmacologic study with which to study these and other ephedrine-like drugs in varying dosage using larger series of subjects.

TABLE V

RESULTS OF CLINICAL TRIAL

DRUGS (ORALLY)*	CASES HELPED	CASES NOT HELPED	SUBJECTIVE SYMPTOMS	CASES HELPED BY BOTH DRUGS	CASES HELPED BY NEITHER DRUG	NO. 1 BETTER THAN NO. 2	NO. 1 AND NO. 2 EQUALLY GOOD	NO. 2 BETTER THAN NO. 1
1	19	13	2	11	7	3	14	5
2	25	10	2					

RESULTS BY SYMPTOMS

SYMPTOM AND NUMBER OF CASES	DRUG* (ORALLY)	CASES	CASES HELPED	CASES NOT HELPED	SUBJECTIVE SYMPTOMS	CASES GIVEN BOTH DRUGS	CASES HELPED BY BOTH	CASES HELPED BY NEITHER	NO. 1 BETTER THAN NO. 2	NO. 1 AND NO. 2 EQUALLY GOOD	NO. 2 BETTER THAN NO. 1
Asthma 26	1	20	12	8	2	12	5	4	2	8	2
	2	18	12	6	1						
Rhinitis 13	1	7	5	2	0	6	4	1	1	3	2
	2	12	10	2	0						
Urticaria 7	1	7	4	3	0	5	3	1	0	3	2
	2	5	4	1	0						
Angioneurotic edema 1	1	1	0	1	0	1	0	1	0	1	0
	2	1	0	1	1						
Migraine 1	1	0				0					
	2	1	1	0	0						

*Drug 1 = Methyl racephedrine hydrochloride.
Drug 2 = Beta-o-methoxyphenyl-n-propyldimethylamine hydrochloride.

ASSAY OF EPINEPHRINE AND EPHEDRINE ANALOGUES

The program of clinical assay as we have developed it includes determination of the pulse rate, blood pressure, and vital capacity before and after hypodermic administration of the test drug, and the effect of the injected material on the evolution or involution of histamine wheals produced either by iontophoresis or intracutaneous injections. In this series we did not study the local effect on the nasal mucosa.

In some of the series vital capacity determinations were supplemented by determinations of the coefficient of ventilation, as described by Brice. The coefficient of ventilation represents the ratio of oxygen consumed per minute to the oxygen respired for a minute. Theoretically, this test has an advantage over the vital capacity test, since, in the former, the patient rests passively, while in the latter active cooperation is required. Determination of the coefficient of ventilation is as follows:

The patient reports in at the same time each day, but not necessarily in the postabsorptive state. After a twenty-minute rest in bed a tracing is made

similar to that for the basal metabolic rate determination. We used the Jones metabolism apparatus. The usual line touching the points of expiration is drawn, as well as a line touching the points of inspiration. The distance between the lines measures the mean respiratory amplitude (MRA) or tidal ventilation. The time required to consume one liter of oxygen is determined, and from this the cubic centimeters of oxygen consumed per minute (O_2C) is calculated. The average respiratory rate (ARR) is obtained by counting the total respirations and dividing this total by the time required for the test. The coefficient of ventilation is then calculated from the formula:

$$CV = \frac{O_2C \times 100}{ARR \times MRA}$$

After completion of the tracing and recording of pulse rate and blood pressure, the patient stands next to the bed and blows into a spirometer for measurement of the vital capacity, after which he again lies down. These determinations are repeated after twenty minutes, after which the drug to be tested is injected subcutaneously. The four determinations, pulse rate, blood pressure, coefficient of ventilation, and vital capacity, are then repeated at twenty-minute intervals for an additional two hours and twenty minutes. The total time required is three hours. To avoid the possibility of cumulative effect, only one drug is tested each day. Each subject was tested with each of the drugs under study.

The response to 0.4 c.c. of 1:1,000 epinephrine as a standard for comparison was studied in nine cases. In seven, determinations were made of response to 50 mg. of ephedrine, 25 mg. of ephedrine, 50 mg. of methyl racephedrine hydrochloride, 50 mg. of beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride, and 50 mg. of beta-o-methoxyphenyl-n-propyl-methylamine hydrochloride.* The time required was not inconsiderable, amounting to 44 seances of three hours each. The results are summarized in Table VI B, which is derived as follows:

The actual determinations are averaged, as illustrated for the case of epinephrine in Table VI A. The first vertical column on Table VI B is derived from Table VI A by subtracting the higher of the two control figures from the highest of the several average figures recorded after the injection of epinephrine. Figures for the other drugs were derived from tables similar to Table VI A.

Epinephrine increased the vital capacity an average of 460 c.c. as compared with 120 c.c. for ephedrine, and 20, 70, and 30 c.c. each for the analogues tested. The coefficient of ventilation was increased most after epinephrine, while the analogues compared favorably with ephedrine in this respect.

As a check on the reliability of these objective methods of study of respiratory efficiency, clinical trial was made by dispensing 25 mg. capsules of the analogues to 70 patients with asthma, 35 with rhinitis, and 19 with urticaria for comparison with each other and with 25 mg. dosages of ephedrine. In no case was the effectiveness superior to that of ephedrine, and in the majority all three analogues were less effective. In comparing the three among themselves some patients believed that one was a little better, while other patients

See footnote, page 261.

selected another. While there was no uniformity beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride was selected a little more often as better than the other two in both asthma and urticaria. Furthermore, it depressed experimental histamine whealing definitely better than the other two, and indeed, slightly more effectively than did ephedrine in the same dosage.

It is our impression that the increase in vital capacity after these several drugs more nearly paralleled their actual effectiveness in relieving asthma than did the change in the coefficient of ventilation. This is fortunate to the extent that vital capacity determination is much less time-consuming than ventilation coefficient determination.

TABLE VI A

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF 0.4 C.C. OF EPINEPHRINE ON THE PULSE RATE, BLOOD PRESSURE, VITAL CAPACITY, AND COEFFICIENT OF VENTILATION (AVERAGE OF 9 CASES)

	CONTROL		MINUTES AFTER INJECTION OF EPINEPHRINE						
	1	2	20	40	60	80	100	120	140
Pulse rate	81.3	81.3	81.4	81.0	81.1	79.4	78.9	78.0	80.0
Blood pressure	118.8	120.1	128.0	125.8	125.8	122.0	117.6	119.9	120.9
Vital capacity	2.49	2.60	2.99	3.06	3.0	3.01	2.74	2.63	2.66
Coefficient of ventilation	2.42	2.58	3.46	3.39	3.43	3.96	3.98	4.20	4.48

TABLE VI B

AVERAGE INCREASE IN PULSE RATE, BLOOD PRESSURE (MM. HG), VITAL CAPACITY (LITERS) AND COEFFICIENT OF VENTILATION AFTER SUBCUTANEOUS TREATMENT WITH TEST DRUGS

	EPINEPHRINE 1:1,000 0.4 C.C.	EPHEDRINE 50 MG.	EPHEDRINE 25 MG.	METHYL RACEPHEDRINE 50 MG.	BETA-O-METHOXYPHENYL-N-PROPYL-*di*-METHYLAMINE 50 MG.	BETA-O-METHOXYPHENYL-N-PROPYL-METHYLAMINE 50 MG.
Pulse rate	0.6	3	1.1	0.1	0.3	1.2
Blood pressure	7.9	10.5	3.2	11.3	5.6	11.9
Vital capacity	0.46	0.12	0	0.02	0.07	0.03
Coefficient of ventilation	1.90	0.41	0.87	0.92	0.76	0.69

As a final check we ran another series of vital capacity determinations on an additional six cases, comparing the response to ephedrine, methyl racephedrine, and beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride, given subcutaneously. Beta-o-methoxyphenyl-n-propylmethylamine hydrochloride was not used in this series, since it had been found clinically to be the least effective of the three. The average increase after ephedrine was 190 c.c.; after methyl racephedrine, 120 c.c.; and after beta-o-methoxyphenyl-n-propyl*di*methylamine hydrochloride it was 250 c.c. This shows the same superiority of the last over methyl racephedrine. When this last series is compared with the results in Table VI B there is a consistency in the findings, indicating that vital capacity determination is an adequate measure of the effectiveness of these drugs in asthma.

Beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride was no more effective clinically than ephedrine, and yet in the last series it was more effective in increasing the vital capacity to the extent of 60 c.c. We conclude that a difference of at least 60 c.c., probably better 75 or 100 c.c. must be present to be significant.

CONCLUSIONS

A program has been devised for the clinical assay of ephedrine analogues. This consists in serial determinations (before and after hypodermic administration of the drug) of pulse rate, blood pressure, and vital capacity; determination of the drug's effect on the evolution and involution of histamine wheals; and study of the local reaction in one side of the nose compared with the other side as a control, with the use of the Glatzel-Mulinos Mirror, as modified by Vaughan and Derbes.

Both epinephrine and ephedrine should be used as comparison controls. Superiority over ephedrine should require an average increase of vital capacity of at least 75 c.c. better than ephedrine.

Results of such objective study should be checked by clinical trial.

Failure of an ephedrine or epinephrine analogue to benefit one allergic symptom or reaction does not necessarily mean that it will be ineffective in all such reactions. Clinical assay should, therefore, evaluate the several types *of response.*

The drugs studied were provided through the courtesy of The Upjohn Research Laboratories where preliminary pharmacologic laboratory investigations had been made.

REFERENCES

Brice, A. T.: Pulmonary Ventilation in Health and Disease, Am. J. Clin. Path. 12: 144, 1942.
Brice, A. T.: New Index of Respiratory Efficiency, Am. J. M. Technol. 5: 11, 1939.
Cameron, W. H., and Kasanin, J.: A Pharmacologic and Clinical Re-evaluation of Amphetamine (Benzedrine) Sulfate, New England J. Med. 224: 544, 1941.
Cori, Carl F., and Welch, Arnold DeM.: Glandular Physiology and Therapy, J. A. M. A. 116: 2590, 1941.
Gaddum, J. H.: Recent Discoveries About Hormones, Pharm. J. 142: 27, 1939.
Goodman, Louis, and Gilman, Alfred: The Pharmacological Basis of Therapeutics, New York, 1941, The Macmillan Company.
Gunn, J. A.: The Pharmacological Actions and Therapeutic Uses of Some Compounds Related to Adrenaline, Brit. M. J. 2: 155, 1939.
Gunn, J. A.: The Pharmacological Actions and Therapeutic Uses of Some Compounds Related to Adrenaline, Brit. M. J. 2: 214, 1939.
Mann, P. J. G., and Quastel, J. H.: Benzedrine (B-phenylisopropylamine) and Brain Metabolism, Biochem. J. 34: 414, 1940.
Myerson, A., Loman, J., and Dameshek, William: Physiologic Effects of Benzedrine and Its Relationship to Other Drugs Affecting the Autonomic Nervous System, Am. J. M. Sc. 192: 560, 1936.
Richards, W. D., Barach, A. L., and Cromwell, H. A.: Use of Vaporized Bronchodilator Solutions in Asthma and Emphysema, Am. J. M. Sc. 199: 225, 1940.
Starr, I., Collins, L. H., and Wood, F. C.: Studies in Basal Work and Cardiac Output of the Heart in Clinical Conditions, J. Clin. Investigation 12: 13, 1933.
Tainter, M. L.: The Sympathomimetic Amines as Epinephrine Substitutes, J. A. M. A. 116: 2769, 1941.
Vaughan, Warren T., and Derbes, Vincent J.: Further Modifications of the Nasal Contact Test for Allergy, Ann. Otol., Rhin. & Laryng. 50: 1141, 1941.
Vaughan, Warren T., and Pipes, David M.: Subcutaneous Tissue Pressure Studies in Urticaria and Angioneurotic Edema, J. Allergy 11: 349, 1940.
Woodruff, E. H., Lambooy, J. P., and Burt, W. E.: Physiologically Active Amines, J. Am. Chem. Soc. 62: 922, 1940.

THE EFFECT OF TESTOSTERONE PROPIONATE IN THE TREATMENT OF ARTERIOSCLEROSIS OBLITERANS*

HERMAN ZURROW, M.D., GAMLIEL SALAND,† M.D., CHARLES KLEIN, M.D., AND STANLEY GOLDMAN, M.D., NEW YORK, N. Y.

THE effects of testosterone on the vascularity of the skin[1] and on muscle tissue metabolism[2, 3] have suggested its use in the treatment of patients with arteriosclerosis obliterans. This is a report of a preliminary study made with the intention of evaluating results in a small series of cases to determine the advisability of further work along these lines.

We studied 23 patients; eight of these were treated with biweekly intramuscular injections of 25 mg. of testosterone propionate; fifteen patients were control cases, receiving biweekly intramuscular injections of 3 c.c. of normal saline. All patients were ambulatory and had had a complete general and peripheral vascular study. The peripheral vascular status was determined on the basis of that study.[4] The patients had received no previous treatment for peripheral vascular disease. All were instructed as to foot hygiene and care, diet, and general measures, as outlined in our clinic routine. All patients were re-examined and studied at approximately three- to six-month intervals, and their peripheral vascular status was determined according to the following criteria:

A. Vascular anatomic status: This was determined by the evaluation of the amplitude of vessel pulsation determined by palpation, oscillometry, temperature changes, rubor on dependency and pallor on elevation, and roentgenograms for arterial calcification. This factor was graded from four plus (severe) to zero (no involvement).

B. Tissue anatomic status: This was determined by the degree of involvement of the superficial and deep tissues, and graded four plus when gangrene was present, three plus when ulceration was present, two plus when infection without gangrene was present, one plus when skin atrophy muscle atrophy, nail changes, or epidermatophytosis were present, and zero when no apparent involvement was present.

C. Rest pain: This refers to pain, other than that of claudication, and includes neuritic pain, paresthesias, coldness, night cramps. This was graded four plus for severe pain, to zero for no pain.

D. Claudication: This was measured by the patient's statement regarding the number of blocks he could walk before pain in calves supervened. He was graded four plus if he could walk only one-half block; three plus if he could

*From the Department of Peripheral Vascular Diseases, the Bronx Hospital, New York City.

Received for publication, June 26, 1942.

†Deceased.

walk one-half to two blocks; two plus, if two to four blocks; one plus, if more than four blocks, but still limited; zero, if no claudication was present.

E. Vascular reserve or capacity for vasodilatation: This was studied by means of the thermal vasodilatation test.[5] Where this test did not produce complete vasodilatation, nerve block was performed for corroboration.[6] Vascular reserve was graded four plus for full dilatation; i.e., a rise in skin surface temperature of the big toe to 30.5° C.; three plus, a 75 per cent rise from the initial temperature to 30.5° C.; two plus, a 50 per cent rise; one plus, a 25 per cent rise; zero, no rise.

F. Functional classification: This represents a summation of the disease factors. Class I, patients with organic vascular disease without symptoms; Class IIa, patients with organic vascular disease with minimal symptoms; Class IIb, patients with organic vascular disease with moderately severe symptoms; Class III, patients with organic vascular disease bedridden because of gangrene, infection, or intractable pain; Class IV, patients with symptoms, but with no demonstrable organic vascular disease.

We are reporting the results of our findings at the end of a three- to seven-month period, an eight- to fourteen-month period, and a fourteen- to eighteen-month period of treatment.

RESULTS

After treatment for three to seven months, four patients treated with testosterone propionate showed no improvement in vascular status; two of these patients were improved in tissue anatomic status, rest pain was lessened in two cases, claudication was improved in two cases, vascular reserve was improved in two cases, and functional status was improved in two cases.

Of the seven control cases observed for this period, four cases were improved in vascular anatomic status; two cases were improved in tissue anatomic status, rest pain; and claudication time were not improved in any case; three cases were improved in vascular reserve; two cases were improved in functional status.

It will be noted that for this period of study results were equivocal.

After eight to fourteen months of treatment with testosterone propionate, none of the six cases studied showed improvement in vascular anatomic status, two were improved in tissue anatomic status, rest pain was unrelieved in any case, four were improved in claudication time, three were improved in vascular reserve, and four were improved in functional status.

Of six control cases, two improved in vascular anatomic status, none improved in tissue anatomic status, none was relieved of rest pain, two were improved in claudication time, three were improved in vascular reserve, and two were improved in functional status.

It will be noted that, for this period, no encouraging results were obtained.

After fourteen to eighteen months of treatment with testosterone propionate, one of the two cases studied was improved in vascular anatomic status, one improved in tissue anatomic status, one in claudication time; rest pain was not present, both improved in vascular reserve, and one improved in functional status.

Of the six control cases, two improved in vascular anatomic status, two improved in tissue anatomic status, none were relieved of rest pain, three were improved in claudication time, in vascular reserve, and in functional status.

It will be noted that, for this period of treatment, results were not encouraging.

SUMMARY

Twenty-three patients suffering from obliterative vascular disease of the lower extremities were studied to note the effect of testosterone propionate on the signs and symptoms of their disease. Eight cases received biweekly intramuscular injections of 25 mg. of testosterone propionate; fifteen patients were observed as controls. The entire study was carried on over a period of eighteen months.

No significant effect was noted in the treated cases with respect to vascular anatomic status, tissue anatomic status, vascular reserve, claudication, rest pain, or functional status, as compared with the control cases.

REFERENCES

1. Edwards, E. A., Hamilton, J. B., and Duntley, S. Q.: Testosterone Propionate as a Therapeutic Agent in Patients With Organic Disease of the Peripheral Vessels, New England J. Med. 220: 865, 1939.
2. Kearns, W. M.: The Clinical Application of Testosterone, J. A. M. A. 112: 2255, 1939.
3. Simonson, E., Kearns, W. M., and Enzer, N.: Effect of Oral Administration of Methyltestosterone on Fatigue in Eunuchoids and Castrates, Endocrinology 28: 506, 1941.
4. Saland, G., Klein, C., Zurrow, H., Gootnick, A., and Katz, A.: Criteria for the Classification and Diagnosis of Peripheral Vascular Disease, Arch. Int. Med. 65: 1035, 1940.
5. Saland, G., Klein, C., and Zurrow, H.: The Thermal Reflex Vasodilatation Test in Peripheral Vascular Disease, Am. Heart J. 17: 581, 1939.
6. Gootnick, A., Saland, G., Klein, C., and Zurrow, H.: Studies on Vasodilatation Tests in Peripheral Vascular Disease, J. Lab. & Clin. Med. 27: 878, 1942.

A STUDY OF NORMAL CARDIAC RESPONSE TO WATER BELOW BODY TEMPERATURE WITH SPECIAL REFERENCE TO A SUBMERSION SYNDROME*

W. W. Tuttle, Ph.D., and Joyce L. Templin, M.A.
Iowa City, Iowa

In many institutions the importance of being able to swim as a means of self-preservation is felt so keenly that everyone is required to acquire an elementary mastery of it. Wherever large groups are required to go into a swimming pool, there are always some who experience reactions so disturbing that they never have a feeling of well-being when submerged† in water. In fact, in some cases, their reactions to submersion are so pronounced that it may appear hazardous to compel them to undertake to learn to swim.

These special cases always place an instructor in a quandary as to what to do. It is always a question whether the person who appears distressed when

*From the Department of Physiology, University of Iowa.
Received for publication, June 29, 1942.

†Submersion as used here does not necessarily mean that a person is completely under water. In all cases the head is out of the water.

submerged in water merely is afraid, or whether he actually has physiologic reactions which might be hazardous were he left unsupervised while attempting to swim.

Each year there are numerous drownings attributed to heart failure, cramps, and exhaustion. Horton and Gabrielson[1] investigated the actual causes and concluded that in all probability many drownings were due, in reality, to a syndrome which they called "hypersensitiveness to cold." As a result of their investigations, they propose two tests which seem adequate for detecting persons who are hypersensitive to cold. By making use of such tests it is proposed that persons who are hypersensitive to cold may be appraised of the fact and can thus avoid unpleasant experiences due to submersion in cold water.

The tests proposed by Horton and Gabrielson are (1) the ice-cube test, and (2) the cardiovascular test. The ice-cube test is the simpler of the two but is less reliable because the systemic reactions are limited. It is done as follows: a cube of ice is held in contact with the forearm of a person in the sitting position for three minutes. If he is hypersensitive *to cold*, a wheal slightly *raised* and surrounded by *a red flare* will *appear* within two to five minutes after the removal of the ice cube.

The cardiovascular test is more complicated, but recommended, because it gives better systemic reactions. The test consists of establishing resting heart rates and blood pressures in the supine position. Then the hand and forearm of the subject are immersed in water at a temperature of 8° to 10° C. for from five to ten minutes. At the end of five minutes of immersion blood pressure and pulse rate are recorded. Then the subject's hand is removed from the bath, and blood pressure and pulse rate are taken every two minutes for the next fifteen to twenty minutes. At the same time observations are made for swelling, hives, color changes, and subjective sensations.

In hypersensitive persons exposure of the hand and forearm to a cold bath causes a definite decrease both in systolic and diastolic blood pressures, accompanied by an increase in pulse rate. Other symptoms of hypersensitiveness are swelling of the submerged hand and a definite mark of demarcation at the water line.

The reactions of hypersensitive people to cold are said to be due to a release of a chemical substance, histamine, from the tissue cells of the body in sufficient concentration to produce histamine reactions. In fact, the reactions of the hypersensitive person can readily be duplicated by the injection of histamine.

A number of studies have been reported on the effects of submersion in water on the physiologic reactions both of diving and land animals.[2] The results of these investigations parallel, to a considerable extent, those obtained in the case of man. When diving animals go from land into the water, they suffer a marked drop in heart rate, provided they are accustomed to the surrounding environment. However, when land animals are forcibly submerged, they do not show a similar reaction. The diving animals are observed to relax in the water, while the land animals fail to do so. Due to the unnatural environment even animals show physiologic, as well as psychologic, behavior which is dangerous to their existence in water.

The reactions of some animals, as well as some human beings, to even partial submersion in water raise the question: Should people be forced promiscuously into swimming pools, even though it is granted that swimming is a fine sport, a healthy exercise, and a useful thing to be able to do?

Researches dealing with water submersion show conclusively that man, when even partially submerged, experiences alterations in physiologic responses, especially those involving the cardiovascular system.

Wells[3] studied the effect of submersion in water of varying temperatures on heart rate. His technique was to place the subject in a supine position in a specially arranged tub in which water temperature could be controlled. He began with a temperature of 65° F. and gradually raised it to 110° F. The heart rate was counted at every 5 degree change in temperature. The results of this study are shown in Table I.

TABLE I

EFFECT OF SUBMERSION IN WATER OF VARYING TEMPERATURES ON THE HEART RATE OF 46 NORMAL MALE SUBJECTS

WATER TEMPERATURE DEGREES F.	HEART RATE PER MINUTE
65	72
70	61
75	68
80	66
85	65
90	64
95	64
100	70
105	87
110	120

The data in Table I show that there is a decrease in heart rate due to submersion until the water temperature approximates the body temperature. From this point on, there is an increase in heart rate as long as there is an increase in the water temperature. Since the temperature of swimming pools is maintained somewhere between 65° and 85° F., it is reasonable to expect that persons who respond normally will show a decrease in heart rate when submerged in a swimming pool.

The results of an experiment by Tuttle and Corleaux[4] are further evidence that the normal reaction to submersion in water is a significant decrease in heart rate. They compared the heart rates of 100 men sitting and standing in their normal environment with their pulse rates sitting and standing when submerged in water. The results of this experiment are shown in Table II. The figures are group means. The rates are for one minute.

TABLE II

MEAN PULSE RATES OF A GROUP OF 100 MEN BEFORE AND DURING SUBMERSION IN WATER OF SWIMMING POOL TEMPERATURE

	MEAN NORMAL PULSE RATE	MEAN PULSE RATE IN POOL	MEAN CHANGE IN PULSE RATE
Sitting	87	76	-11
Standing	98	84	-14

The data in Table II are further evidence that the normal reaction to submersion in water of swimming pool temperature is a decrease in pulse rate.

In seeking to establish the response of normal persons to submersion in water, it has been our experience that there has always been a small percentage of cases who failed to conform to the reactions of the group as a whole. It is the purpose of this investigation to study the reactions of this group who appear to react abnormally to submersion.

In order to have available nonconformants for further study a group of persons, unskilled in swimming, were examined in the class situation. Sixty-eight college women registered in beginning swimming classes at the University of Iowa served as subjects. Their ages ranged from 17 to 25 years.

Early in the instructional period each girl was tested as to the response of her heart to submersion. First, a resting pulse rate in the standing position was recorded. Following this, she climbed into the swimming pool and waded into water of shoulder depth. After three minutes her resting pulse rate was recorded again. The count was continued at two-minute intervals as long as there was a change in pulse rate, or until a constant rate was established.

Of the group of 68 women, 7 failed to show a decrease in heart rate while submerged in water and 61 responded normally. A summary of the data collected from the normal group is shown in Table III. The figures are group means per minute.

TABLE III

CARDIAC RESPONSE OF 61 NORMAL SUBJECTS TO SUBMERSION IN WATER

MEAN RESTING RATE STANDING (PER MINUTE)	MEAN RESTING RATE STANDING—SUBMERGED (PER MINUTE)	DIFFERENCE
90	77	-13

The data in Table III show that, on the average, there was a decrease of 13 beats per minute, which is practically the same as that reported for men. The mean decrease amounts to 14 per cent for women as compared to 12 per cent for men.

Table IV shows the relationship between resting pulse rate and the decrease caused by submersion.

TABLE IV

RELATION OF RESTING PULSE RATE TO THE DECREASE IN RATE CAUSED BY SUBMERSION IN WATER

RESTING PULSE RATE (PER MINUTE)	DROP DUE TO SUBMERSION
70-79	5
80-89	11
90-99	14
100-109	16

The data show that the extent of the decrease in pulse rate due to submersion in water varies directly with the resting pulse rate.

The group of 7 girls who failed to respond normally to submersion in water were studied further. There were two possible explanations for the abnormal response of this group: (1) there was lack of psychologic adjustment; that is, they were afraid. In the case of adjustment to submersion in water, fright alone is sufficient to account for a failure to adjust normally; that is, experience a fall in heart rate. (2) There was lack of physiologic adjustment,

Ordinarily, one can expect that repetition of exposure to a strange environment will condition a person so that he will exhibit normal behavior. On the other hand, it is reasonable to suppose that if abnormal response is due to the failure of some physiologic mechanism to adjust itself, repetition will fail to cause an appreciable alteration in the response.

The submersion experiment was repeated on the 7 subjects who failed to experience a decrease in heart rate during submersion. In the interim between the first test and the retest, each member of this group had participated in six or more class hours of swimming instruction, during which they were submerged in water. One would expect this amount of repetition to condition a person to the new environment. The results of the retest are shown in Table V.

Table V

Results of a Retest of Subjects Who Failed to Show Normal Reactions to Submersion in Water

Subject No.		Resting Pulse Rate (per minute)	Pulse Rate While Submerged (per minute)	Difference
18	First test	82	80	- 2
	Retest	86	74	-12
32	First test	92	90	- 2
	Retest	96	90	- 6
53	First test	78	78	0
	Retest	80	76	- 6
23	First test	68	82	+14
	Retest	72	76	+ 4
28	First test	80	80	0
	Retest	72	72	0
43	First test	86	90	+ 4
	Retest	82	92	+10
56	First test	86	88	+ 2
	Retest	88	88	0

The data in Table V show that 3 of the 7 subjects originally classed as abnormal became conditioned to submersion. Subject 18 behaved as a psychologic case, and after sufficient exposure to a water environment responded normally. Subjects 32 and 53 became conditioned to the water to such an extent that there was a significant fall in pulse rate due to submersion.

However, 4 of the 7 cases (23, 28, 43, and 56, Table V) failed completely to become conditioned. They never experienced a decrease in heart rate due to submersion, and it was suspected that they were failing to make normal physiologic adjustments to submersion.

Although the water in the swimming pool was at a temperature between 75° and 85° F. and could not be considered cold, it was decided to subject the nonconditioned group to a test for hypersensitiveness to cold. The cardiovascular function test was given to each, and all of them were hypersensitive to cold according to the criteria suggested by Horton and Gabrielson.

Although it would seem that this group of persons had been exposed to submersion a sufficient number of times so that they should be psychologically conditioned to it, it was decided to gather additional data relative to this point.

For this purpose, each of the subjects (23, 28, 43, and 56) was given a bathtub test. The procedure was to place each person in a bathtub in a sitting position and determine the resting heart rate. Then she sat at the side of the tub while it was filled with water of the temperature of that of the swimming pool. She returned to the tub and after three minutes of submersion the resting pulse rate was taken again. Under these conditions the resting pulse rate was established as previously described while the subject was submerged. The data obtained are shown in Table VI.

TABLE VI

EFFECT ON HEART RATE OF SUBJECTS BEING SUBMERGED IN WATER IN A BATHTUB

SUBJECT NO.	RESTING PULSE RATE (PER MINUTE)	PULSE RATE SUBMERGED (PER MINUTE)	DIFFERENCE
23	66	68	+2
28	68	70	+2
43	66	66	0
56	64	64	0

The data in Table VI show results similar to those obtained in the previous experiments. The data show that, although these subjects did not experience as great an increase in heart rate when submerged in the tub as in the pool, they failed to show any tendency toward a decrease. This lack of physiologic adjustment on the part of some persons may account for their being uncomfortable in the swimming pool, and also the difficulty experienced by them in learning to swim.

CONCLUSIONS

On the basis of data collected from 68 college women, the following conclusions are drawn concerning the effects of submersion in water:

1. Submersion in water of swimming pool temperature causes a drop in the heart rate of normally adjusted persons.
2. The amount of the decrease in heart rate due to submersion varies directly with the resting heart rate.
3. Failure to experience a decrease in heart rate when submerged in water below body temperature is due either to a lack of emotional adjustment (fear) or to a failure to compensate physiologically.
4. Where emotional factors are controlled, failure to experience a significant drop in pulse rate during submersion in water below body temperature indicates sensitivity to the water.
5. It is suggested that the conditions causing a failure to make normal adjustments to submersion in water be called the "submersion syndrome."

REFERENCES

1. Horton, B. T., and Gabrielson, M. A.: Hypersensitiveness to Cold: A Condition Dangerous to Swimmers, Res. Qr. 11: 119, 126, 1940.
2. Irving, L.: Respiration in Diving Animals, Physiol. Rev. 19: 112, 1939.
3. Wells, G.: Effect of External Temperature Changes on Heart Rate, Blood Pressure, Physical Efficiency, Respiration, and Body Temperature, Res. Qr. 3: 108, 1932.
4. Tuttle, W. W., and Corleaux, J. F.: The Response of the Heart to Water of Swimming Pool Temperature, Res. Qr. 6: 24, 1935.

ETIOLOGY AND SERUM TREATMENT OF PERSISTENT EPIDEMIC AND POSTOPERATIVE HICCUP*

EDWARD C. ROSENOW, M.D., ROCHESTER, MINN.

PREVIOUS reports have been made[6, 7, 9, 11] on the consistent isolation from atria of infection of patients suffering from epidemic hiccup, of alpha or green-producing streptococci with which spasms of the diaphragm and other muscles (myoclonic encephalitis) have been produced experimentally in animals. Paroxysms of spasms of the diaphragm, sometimes associated with audible hiccup, were reproduced consistently by direct injection of suspensions of nasopharyngeal swabbings and filtrates of these and with pure cultures of the living streptococcus freshly isolated in dextrose-brain broth, as well as with the corresponding washed, heat and formalin-killed organisms and with filtrates of active cultures.[8] Characteristic symptoms developed in from twenty-four to seventy-two hours after inoculation of relatively small numbers of the living streptococci, in from four to twelve hours after inoculation of large numbers of the killed organisms, and in two to five hours after inoculation of Berkefeld N filtrates. The symptoms after inoculation of the living streptococci lasted the longest and often progressed to death in forty-eight to ninety-six hours; those that occurred after inoculation of the dead streptococci lasted usually from six to twenty-four hours, and those following inoculation of filtrates lasted two to six hours and then disappeared.

Cultural characteristics, virulence, cataphoretic velocity, and antigenicity of the streptococci resembled those of streptococci isolated in studies of epidemic encephalitis. Vaccines prepared from the spasm-producing streptococci protected rabbits against living cultures, the dead organisms, and active filtrates.[8] The streptococci were agglutinated specifically by the encephalitis antistreptococcic serum. This antiserum was found to neutralize specifically the "toxin" or poison produced by the streptococcus of persistent hiccup and to have striking curative effects, under controlled conditions, in animals in which spasms of the diaphragm were produced experimentally.[8] Moreover, the encephalitis antistreptococcic serum appeared curative in the treatment of patients suffering from epidemic and postoperative hiccup.[5, 9]

The occurrence of encephalitis and hiccup simultaneously or in sequence and the experimental production of lesions in animals, corresponding to those responsible for characteristic symptoms in patients, with the respective streptococci freshly isolated from atria of infection of patients having diverse manifestations during a changing epidemic from hiccup to neuromyeloencephalitis,[10] indicate that the inciting agent of these diseases is similar.[1-3, 10] Moreover, the

*From the Division of Experimental Bacteriology, the Mayo Foundation, the Mayo Clinic, Rochester.

Received for publication, June 29, 1942.

changes in localizing power of the streptococci, as isolated from persons, and in this study also from milk and air, on artificial cultivation and on repeated passage through animals indicate that the changes in type of disease in this and other outbreaks studied were due to acquired specificities of streptococci normally present in atria of infection of well persons and in nature generally in nonepidemic times.

The study of the problem of epidemiologic characteristics, causation, and serum treatment of persistent epidemic hiccup has been extended to that of persistent postoperative hiccup. In this new series of cases, as in those previously reported, the cases studied culturally, and those in which serum was used in treatment, occurred chiefly among patients of the Mayo Clinic. The historical aspects of this strange disease, its classification, its reproduction in animals, and its results of serum treatment in a previous series of cases have been set forth by C. W. Mayo. I am indebted to members of the staff and to physicians in widely separated regions of the United States for their cooperation in permitting me to study cases and for reports of results from the use of the serum in cases of intractable hiccup.

METHODS

A diagnostic cutaneous test[4] was made by injecting intradermally on the forearm of the patient 0.03 c.c. of a 10 per cent solution of the euglobulin fraction of antiserum prepared with the streptococci isolated in studies of encephalitis and, as controls, the englobulin of other antisera. The size of the maximal flare of erythema, which occurred in from five to ten minutes, was outlined with pen and ink and from this the area of erythema in square centimeters was determined.

Precipitation tests[4] were made with the whole encephalitis and other antistreptococcic sera and cleared extracts of nasopharyngeal swabbings and the serum of patients, and agglutination tests[4] were made with the respective antisera and convalescent sera and the streptococci as isolated and after passage through animals. Dilutions of 1:10, 1:50, 1:250. and 1:1,250 of the respective antistreptococcic sera, and dilutions of 1:20, 1:40, 1:80, and 1:160 of convalescent and control human sera were used in agglutination tests. Mixtures of respective diluted sera and suspensions of streptococci that had been grown in dextrose-brain broth and preserved in dense suspension in glycerin (2 parts) and 25 per cent solution of sodium chloride (1 part) were incubated at 50° C. for eighteen to twenty-four hours when readings were made, instead of incubating at 37° C. for one and a half hours and then placing in the refrigerator overnight.

Material for cultures and inoculation of animals was obtained chiefly from swabbings of nasopharynges, from pus expressed from tonsils or aspirated from pyorrhea pockets, from urine, from pus expressed from prostates, from samples of stool, and in postoperative cases, in addition, from localized infections, if present, such as appendiceal abscess and infected gall bladder. Cultures were made on blood agar plates and in dextrose-brain broth.

Routinely two rabbits, in special instances as many as ten rabbits per case, and in some instances guinea pigs, were inoculated intracerebrally with 0.1 and

0.2 c.c., respectively, of the suspension of material *directly from the naso*pharynx, tonsils, and so forth, suspended in 2 c.c. of gelatin (0.2 per cent) Locke's solution, or with these amounts of pure cultures of the streptococcus in dextrose-brain broth, diluted in dextrose-brain broth 1:200 or 1:10,000 immediately before injection. In special instances, much higher dilutions (as *high* as 1:100,000,000) of cultures were given intracerebrally, and undiluted cultures were given intravenously and intratibially to rabbits. Monkeys and dogs were inoculated intracerebrally with from 1 to 5 c.c. of cultures diluted 1:200 to 1:10,000. Five-tenths cubic centimeter to 1 c.c. of suspensions of the heat-killed streptococci, ten times the density of broth cultures, or of filtrates of cultures, was inoculated intracerebrally into rabbits.

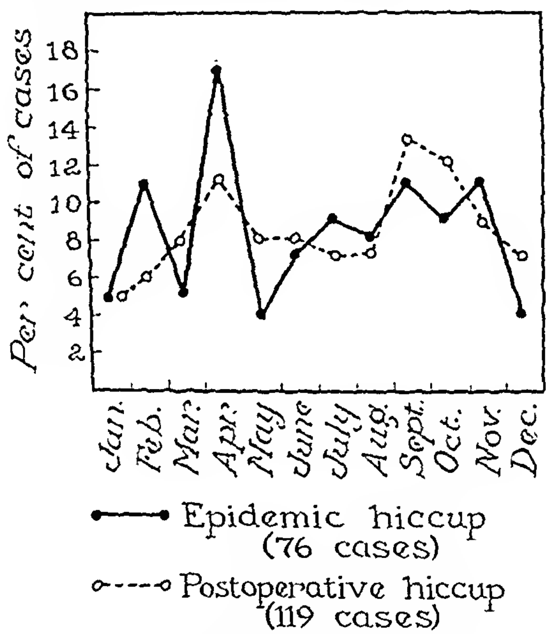


Fig. 1.—Percentage incidence by months of cases of epidemic and postoperative hiccup, Mayo Clinic, 1927 to 1941.

Pure cultures of the streptococcus were obtained from mixtures by making subcultures in rapid succession in previously warmed dextrose-brain broth, and from the end point of growth in serial dilution cultures[12] alternately in dextrose-brain broth and soft dextrose-brain agar (0.2 per cent dextrose and 0.2 per cent agar) and not by plating on blood agar. One or a few additional platings on blood agar usually destroyed the property on which elective localization depends, whereas this property was maintained at the end point of growth of streptococci in serial dilution cultures and after as many as 75 rapidly repeated subcultures in dextrose-brain broth.

Animals were observed two or more times daily after inoculation and their symptoms were recorded. Necropsies were performed as soon as possible after death from the inocula or from ether. Cultures from pipettings of the brain, spinal cord, cerebrospinal fluid, and blood were made routinely in dextrose-brain broth and on blood agar plates. In special instances cultures were made from liver, spleen, kidney, adrenals, muscles, and nerves. Tissues were put aside in 10 per cent solution of formalin for microscopic study.

Cultures were made from the air of rooms occupied by persons having persistent hiccup by exposing open tubes of dextrose-brain broth or by bubbling the air through dextrose-brain broth, chick-mash medium, and dextrose broth for twenty-four to forty-eight hours. For precipitation tests sterile distilled water was likewise exposed in shallow layers in Petri dishes or air was bubbled through water contained in tall columns in test tubes.

SEASONAL INCIDENCE OF EPIDEMIC AND POSTOPERATIVE HICCUP

The occurrence of cases of persistent hiccup in unusual numbers at certain seasons was noted throughout the years of this study (1927 to 1941). There were great variations in severity and duration of the disease and accompanying gastric distress and other symptoms. The great majority of cases studied occurred in men from 17 to 82 years of age. In most instances attacks followed mild, and less often severe, respiratory infections. The seasonal and simultaneous occurrence of epidemic and postoperative hiccup in relation to relatively mild outbreaks of respiratory infections in spring and autumn is well illustrated in Fig. 1.

TABLE I

ERYTHEMATOUS REACTIONS OF PERSONS HAVING PERSISTENT HICCUP TO INTRADERMAL INJECTION OF THE EUGLOBULIN FRACTION OF ENCEPHALITIS AND OTHER ANTISTREPTOCOCCIC SERA

DISEASE GROUPS	CASES	ERYTHEMATOUS REACTIONS TO:						
		EUGLOBULIN FROM STREPTOCOCCIC ANTISERA						NORMAL HORSE SERUM
		ENCEPHALITIS	POLIOMYELITIS	EPILEPSY	ARTHRITIS	INFLUENZA	ULCERATIVE COLITIS	
Epidemic and postoperative hiccup	20	32* / 6:69	8 / 4:25	10 / 5:50	18 / 3:22	10 / 5:40	12 / 2:50	18 / 1:0
Epidemic encephalitis	32	66 / 7:68	20 / 5:55	8 / 6:63	25 / 5:36	19 / 5:53		30 / 1:0
Acute influenza	32	25 / 1:20	7 / 1:0		15 / 1:26	32 / 12:91	10 / 1:50	8 / 0.5:0
Normal controls	84	91 / 1:13	93 / 1:8		81 / 2:13	77 / 1:17	135 / 1:7	84 / 1:0

*The figures above the line in each instance indicate the number of tests; the two sets of figures below the line indicate, respectively, the average reaction in square centimeters and the percentage of reactions measuring 5 sq. cm. or more. Fractions are given to the nearest whole number.

RESULTS OF CUTANEOUS AND PRECIPITATION TESTS

The results from intracutaneous inoculation of persons having persistent epidemic or postoperative hiccup with solutions of the water insoluble or euglobulin fraction of the serum of horses immunized with streptococci isolated in studies of encephalitis, poliomyelitis, epilepsy, arthritis, influenza, and ulcerative colitis are summarized in Table I. The reactions indicate a close relation between the infecting agents of epidemic and postoperative hiccup and epidemic encephalitis. Both the average reaction and the percentage of reactions 5 sq. cm. or more in size in these two groups of cases were greatest to the encephalitis euglobulin, second greatest to the epilepsy euglobulin, third greatest to the influenza euglobulin, and least to the poliomyelitis and arthritis euglobulins. The average reaction and percentage of reactions of 5 sq. cm. or more in the group of cases of influenza were extremely high to the influenza

englobulin and low to the other englobulins. The average reaction and incidence of reactions of 5 sq. cm. or more to the different englobulins were consistently low in the group of normal controls.

The results of precipitation reactions between the encephalitis and other antistreptococcic sera and cleared extracts of nasopharyngeal swabbings and sera of persons having epidemic or postoperative hiccup are summarized in Table II. In accord with epidemiologic and clinical observations, the incidence of positive reactions with sera and extracts of nasopharyngeal swabbings of patients having persistent hiccup was strikingly high with the encephalitis antiserum and moderately so with influenza and epilepsy antistreptococcic sera. Likewise, in the groups of cases of encephalitis and influenza, the reactions were high with the respective homologous antisera and consistently low in the control group of well persons.

TABLE II

PRECIPITATION REACTION BETWEEN ENCEPHALITIS AND OTHER ANTISTREPTOCOCCIC SERA AND CLEARED EXTRACTS OF NASOPHARYNGEAL SWABBINGS AND SERA OF PATIENTS

ANTIGENS	DISEASE GROUPS	CASES	EXTRACTS OR SERA	PERCENTAGE INCIDENCE OF PRECIPITATION* BY STREPTOCOCCIC ANTISERA FOR					
				ENCEPHALITIS	POLIOMYELITIS	ARTHRITIS	INFLUENZA	ULCERATIVE COLITIS	EPILEPSY
Extracts of nasopharyngeal swabbings	Epidemic and postoperative hiccup	22	26	61	23	35	65	54	73
	Epidemic encephalitis	114	114	78	21	5	0	0	
	Acute influenza†	70	70	16	14	34	69	20	34
	Well persons remote from epidemics	60	68	4	0	3	4	6	0
Sera of patients	Epidemic and postoperative hiccup	24	30	37	0	0	27	10	33
	Epidemic encephalitis	67	67	73	25	0	0	0	
	Acute influenza	22	21	0	0	8	22		
	Well persons remote from epidemics	108	108	4	2	6	5	2	0

*No precipitation occurred with normal horse serum.

†The term "influenza" in this study is used to designate the more severe type of seasonal epidemic respiratory infections.

AGGLUTINATION OF THE STREPTOCOCCUS

The results of agglutination tests with streptococci isolated from atria of infection in cases of epidemic and postoperative hiccup, encephalitis and influenza, and from well persons remote from epidemics are summarized in Table III. A high incidence of specific agglutination occurred with the encephalitis antistreptococcic serum and streptococci isolated from atria of infection of persons having persistent hiccup, and interestingly, the incidence was relatively high with antisera prepared with the closely related streptococci isolated in studies of influenza and the spasm-producing streptococci isolated in studies of epilepsy. Streptococci isolated in studies of encephalitis and influenza were specifically agglutinated by the respective homologous antisera, whereas control strains from well persons were not.

TABLE III

AGGLUTINATION BY ENCEPHALITIS ANTISTREPTOCOCCIC SERUM OF STREPTOCOCCI ISOLATED DURING STUDIES OF EPIDEMIC AND POSTOPERATIVE HICCUP

SOURCE OF STREPTOCOCCI	CASES OR STRAINS	CULTURES	PERCENTAGE INCIDENCE OF SPECIFIC AGGLUTINATION* BY STREPTOCOCCIC ANTISERA FOR					
			ENCEPHALITIS	POLIOMYELITIS	ARTHRITIS	INFLUENZA	ULCERATIVE COLITIS	EPILEPSY
Epidemic and postoperative hiccup	40	49	45	0	0	16	0	39
Epidemic encephalitis	96	144	75	9	10	0	0	3
Epidemic influenza	84	84	0	5	5	57		24
Well persons remote from epidemics	49	49	8	4	22	14	0	16

*No agglutination with normal horse serum occurred with any of the suspensions and in some agglutination was nonspecific.

As has been shown, encephalitis streptococcic antigen was demonstrated in the serum by precipitation tests (Table II) and in the skin by intradermal injection of antibody (Table I) during the acute stage of epidemic and postoperative persistent hiccup. As recovery occurred, promptly following therapeutic injection of the encephalitis antistreptococcic serum, and more slowly without serum treatment, the reactivity of serum and skin (antigen) disappeared as specific antibodies became demonstrable for strains of streptococci obtained in studies of hiccup and encephalitis. The results of agglutination tests with convalescent serum are well illustrated by the following observations.

Each of nine sera obtained from persons convalescent from hiccup agglutinated to a greater degree and in higher dilution seven of nine strains of streptococci isolated in studies of epidemic and postoperative hiccup than did nine control sera obtained from patients having arthritis, fibrositis, or influenza. The nine convalescent sera did not agglutinate composite suspensions of streptococci isolated in studies of arthritis, epilepsy, and influenza, respectively. In sharp contrast, the sera from persons having arthritis, epilepsy, or influenza agglutinated specifically the composite suspensions of streptococci isolated, respectively, in studies of these diseases.

ANIMAL EXPERIMENTS

The results obtained on intracerebral inoculation of the living streptococcus, the heat-killed streptococcus, and filtrates of cultures of streptococci in relation to persistent hiccup are summarized in Table IV. It will be noted that the incidence of hyperpnea (49 to 100 per cent) and of spasms of the diaphragm (62 to 88 per cent) and other muscles (65 to 90 per cent) was extremely high, and the incidence of flaccid paralysis (0 to 13 per cent) was very low after inoculation of the living streptococci, corresponding dead streptococci, and filtrates of cultures obtained in relation to hiccup. In contrast, the incidence of spasms of the diaphragm (2 per cent) and other muscles (19 per cent) after inoculation of streptococci from well persons and from indoor air of rooms of persons having poliomyelitis (0 and 57 per cent, respectively) was low. The incidence of flaccid paralysis was extremely low (6 per cent) after inoculation of the streptococci from the group of well persons, and very high (81 per cent) after inoculation of streptococci from indoor air in relation to

TABLE IV

RESULTS IN RABBITS AFTER INTRACEREBRAL INOCULATION OF THE LIVING STREPTOCOCCUS, FILTRATES OF CULTURES, AND THE HEAT-KILLED STREPTOCOCCUS, ISOLATED IN STUDIES OF EPIDEMIC AND POSTOPERATIVE HICCUP

SOURCE OF STREPTOCOCCI AND FILTRATES			STRAINS	ANIMALS INJECTED	SYMPTOMS (PER CENT)				ANIMALS THAT DIED								
					HYPERPNEA	SPASMS		FLACCID PARALYSIS	NUMBER	PER CENT	PER CENT WITH LESIONS OF:			CULTURES YIELDING STREPTOCOCCI			
														BRAIN		BLOOD	
						DIAPHRAGM	OTHER MUSCLES				DIAPHRAGM	LUNG	STOMACH	CULTURES	PER CENT	CULTURES	PER CENT
Living streptococcic from:	Nasopharynx	Epidemic hiccup	40	128	63	62	65	3	74	58	24	42	39	91	86	81	2
		Postoperative hiccup	35	89	49	71	81	0	47	53	15	62	34	63	73	39	5
		Influenza during outbreak of hiccup	18	21	90	62	67	5	19	90	32	68	21	12	100	12	67
		Well persons remote from epidemic hiccup (controls)	44	64	25	2	19	6	27	42	0	48	15	40	53	22	9
	Indoor air, cases of epidemic hiccup		5	15	80	73	87	0	13	87	0	62	31	12	75	12	0
	Indoor air, cases of epidemic poliomyelitis		10	21	29	0	57	81	18	86	0	67	0	15	73	13	0
	Pasteurized and raw milk supplies, cases of persistent hiccup		10	30	50	80	90	13	10	33	40	60	30	8	63	8	0
Nasopharynx, postoperative hiccup	Filtrates of nasopharyngeal washings		9	16	88	81	81	0	0	0	0	0	0	0	0	0	0
	Filtrates of dextrose-brain broth cultures		7	10	90	70	70	0	0	0	0	0	0	0	0	0	0
	Suspensions of heat-killed streptococci		7	16	100	88	88	0	0	0	0	0	0	0	0	0	0

cases of poliomyelitis. Specific effects were especially striking after inoculation of filtrates and suspensions of heat-killed streptococci, when ultimate dosage could be controlled better than by inoculating the living streptococci.

The mortality rate varied greatly after inoculation of living streptococci, being highest in animals that received the streptococci from patients having influenza (90 per cent) and in those that received streptococci from air of rooms of persons having epidemic hiccup (87 per cent) or poliomyelitis (86 per cent); intermediate after inoculation of streptococci from throats of patients suffering from epidemic hiccup (58 per cent) or postoperative hiccup (53 per cent), or of well persons remote from epidemics (42 per cent); and lowest (33 per cent) after inoculation of streptococci from milk supplies.

Lesions of the diaphragm, consisting of localized hemorrhages, occurred only in animals that had violent spasms after inoculation of streptococci isolated from persons having persistent hiccup.

Lesions of the lungs, consisting of hemorrhagic edema and bronchopneumonia, occurred most often and, with one exception, were most conspicuous after inoculation of the streptococcus isolated from persons having persistent hiccup or from milk and air in relation to persistent hiccup and influenza. In accord with the frequent occurrence of gastric distress in patients, lesions of the stomach were common and consisted of numerous, small, superficial hemorrhages in the mucous membrane apparently due to violent spasms, and of early, often almost immediate, post-mortem digestion of the wall of the stomach.

The incidence of isolation of streptococci from the brain, the site of inoculation, was relatively high in each group of cases, whereas the incidence of their isolation from blood of animals that died was very low (0 to 9 per cent), with the exception of those inoculated with streptococci from the nasopharynges of persons having acute influenza (67 per cent).

The spasm-producing type of streptococcus was isolated repeatedly from some patients during acute attacks of hiccup but not after recovery. Its presence was demonstrated in persons during recurring attacks over periods as long as four years, in atria of infection in persons having mild but continuous symptoms for as long as two years, and in several protracted cases in which the attending physician considered the spasms of functional origin. In some cases it was isolated from nasopharynx, infected teeth, tonsils, prostatic secretion, urine, stool, or pus from appendiceal abscesses and infected gall bladders.

MICROSCOPIC LESIONS OF THE BRAIN AND SPINAL CORD OF EXPERIMENTAL ANIMALS

Sections of the brain and spinal cord were made and examined for lesions in altogether 39 animals that died from inocula or that were anesthetized after inoculation. The duration of the experiment in these animals was one to two days in 14; three to five days in 15, and six days or longer in 10. Suspensions in gelatin-Locke's solution of nasopharyngeal swabbings and other material directly from patients were inoculated in 12 animals, cultures of living streptococci in 21, dead streptococci in two, and filtrates in four.

The lesions found in animals that died within one to two days after inoculation consisted chiefly of a relatively mild, but never grossly suppurative, polymorphonuclear meningitis, usually most pronounced over the anterior aspect

of the medulla. Perivascular, leucocytic infiltration, degeneration, edema and localized hemorrhages in sulci, choroid plexus, and anterior aspect of the me-

Fig. 2.—Lesions in rabbits in which spasms of the diaphragm developed after intracerebral inoculation of living streptococci isolated from the nasopharynx of a patient having epidemic hiccup: *a*, in the medulla; *b*, in the brain of a rabbit five days after inoculation; *c*, in the medulla of another rabbit three days after inoculation of the same culture (hematoxylin and eosin, ×110).

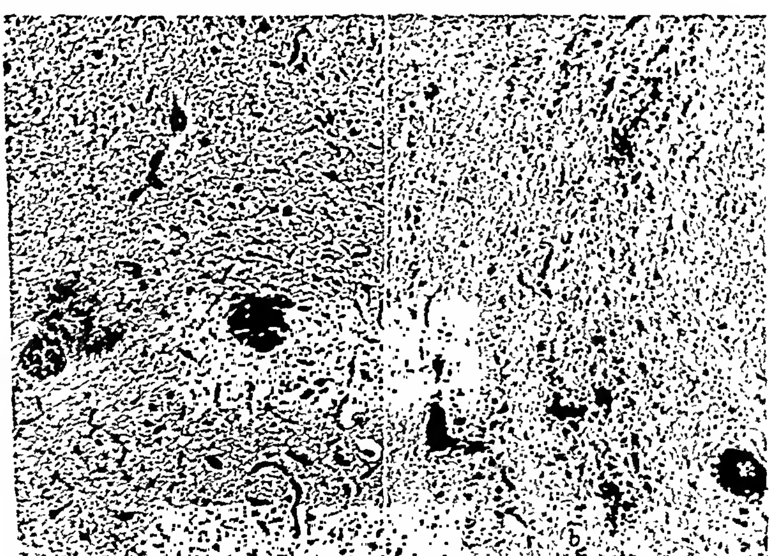


Fig. 3.—Lesions in animals in which spasms of the diaphragm had developed after intracerebral inoculation of living streptococci from the nasopharynges of patients having postoperative hiccup: *a*, in the medulla of a guinea pig six days after inoculation; *b*, in the pons of a rabbit five days after inoculation (hematoxylin and eosin, ×110).

dulla were often conspicuous two days after inoculation of material containing living streptococci, dead streptococci, and active filtrates. Following this, there was a rapid transition from polymorphonuclear cells to lymphocytes,

especially surrounding blood vessels in the pons, floor of the fourth ventricle, medulla, subcortical region, and choroid plexus. This transition in type of infiltrating cells occurred earlier within the parenchyma than in the meninges (Fig. 2). After five or six days typical lymphocytic infiltration, degeneration of ganglion cells (Fig. 3), circumscribed regions of glial infiltration, and other lesions of encephalitis were found. In no instance were the lesions characteristic of spontaneous encephalitis encountered. Necrosis associated with leucocytic and lymphocytic infiltration at the point of injection in the right frontal lobe occurred commonly, but abscess formation was present only occasionally. The lesions remote from the point of injection were essentially alike on both sides. The streptococci were readily demonstrable, especially as diplococci, in the lesions of animals that died soon after inoculation. As lymphocytic infiltration became marked, their demonstration became difficult and often impossible (Fig. 4).

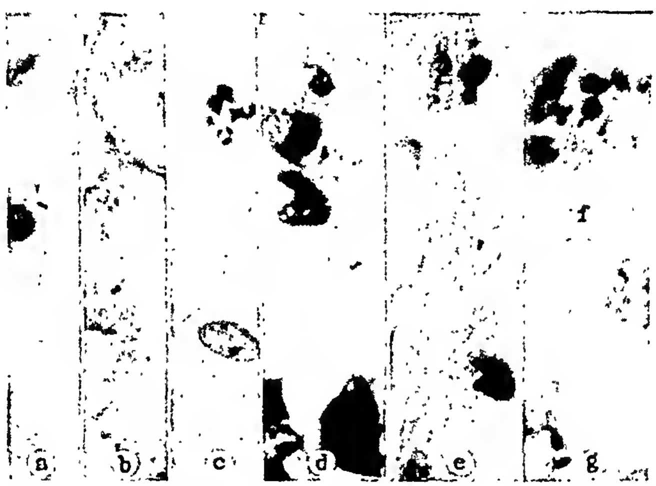


Fig. 4.—Diplococci in the lesions of rabbits and guinea pigs in which spasms of the diaphragm had developed after inoculation of living streptococci (*a*, *b*, *c*, *d*, and *f*) and heat-killed streptococci (*e* and *g*) isolated in studies of epidemic and postoperative hiccup (modified Gram stain, ×1,000).

SERUM TREATMENT

Serum treatment was given only after the usual methods, such as administration of carbon dioxide, gastric lavage, and sedatives, to control the spasms had proved of no avail. The whole, unconcentrated encephalitis antistreptococcic serum was used at first, whereas later the refined serum of similar antibody content, consisting of solutions of one part of the euglobulin and two parts of the pseudoglobulin, was used. After determining that the patient was not allergic to horse serum, and after desensitization if he was found sensitive, 10 c.c. of the serum were injected deeply into the muscles of the buttock twice daily until the hiccup had disappeared, and usually one additional injection was given after that. The concentrated encephalitis antiserum and the euglobulin solution for the cutaneous test, first prepared by me, are now being made available for study by the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

Records of results from the use of encephalitis antistreptococcic serum in treatment have been obtained in altogether 90 cases of persistent hiccup. Fifty-four of these were considered as cases of epidemic persistent hiccup and 36 occurred postoperatively. The results from the use of the serum were so similar in the two groups of cases that they will be considered as one group.

The ages of the patients ranged from 7 to 82 years. The great majority were more than 50 years of age. In all but two postoperative cases, and all but six of the nonoperative cases the patients were men, a proportion of approximately ten men to one woman. The duration of the disease at the time of the first serum treatment was from one to twenty-one days in acute cases, as long as two years in persons having continuous hiccup, and four years in those having recurring attacks. The amount of serum given ranged from 10 to 110 c.c., usually 20 to 40 c.c.

Detailed records regarding noteworthy diminution of severity and number of spasms and cessation of hiccup in relation to the number of injections of serum were available in 60 cases. Reduction of severity and number of spasms was noted after the first injection of serum in 20 cases, after two injections in 21 cases, and after three or more injections in 12, whereas in seven cases no apparent benefit followed soon after injections of serum. Cessation of spasms occurred after the first injection in 14 cases, often within several hours; after two injections in 16 cases, after three injections in nine, and after four injections in 10 cases. In the remaining seven cases in which the patients survived, the hiccup disappeared after from seven to ten days during the period of sensitization. In four postoperative cases no apparent beneficial effects were noted after the administration of the serum. Two of these patients died: one, a man 78 years of age, from cardiac failure following suprapubic prostatectomy in the presence of a badly infected bladder; the other, a man 72 years of age, from peritonitis following strangulated hernia. In the other two, hiccup occurred after prostatectomy, continued for two weeks, and then gradually disappeared.

Immediate serum reactions did not occur in any case. In 15 there were late serum reactions and of these only five were severe.

In the 36 postoperative cases 11 patients had had prostatectomy; two, cystoscopy; five, appendectomy with appendiceal abscess; three, colostomy; seven, cholecystectomy; two, herniotomy; one, gastrectomy; two, rectal resections for carcinoma; one, nephrotomy; one, septic hip; and one, tonsillectomy. There was great variation in the effects of the serum after its injection. The sudden permanent cessation from one or two injections, after every other known means had failed to give relief, often in patients severely exhausted, was so convincing as to leave little doubt of the curative action of the serum. The earlier the serum was given, the more prompt was the relief. The cessation of hiccup after administration of the serum was not coincidental because usually more injections were necessary late in the course of attacks than when given early. Prompt relief after administration of the serum was often associated with striking diminution in reactivity of the skin on reinjection of the encephalitis euglobulin and a negative precipitation reaction with the patient's

serum. This was true of both the nonoperative and operative cases. These observations are in accord with the results of treatment reported by Mayo, assisted by Rozendaal, who stated:

"That there is a relationship between the organisms of epidemic encephalitis and of epidemic hiccup is fortunate, because the encephalitis antibody globulin solution as prepared by Rosenow can be applied in treatment. Such treatment was given in those of our cases in which persistent hiccup was present, and we were able to recover the neurogenic streptococcus from the throat and reproduce spasms in animals. The effect of the serum was dramatic in many instances, all symptoms of hiccup disappearing within a few hours."

Most of the patients whom I saw had badly infected teeth or other evident foci of infection. There was definite correlation between focal and metastatic infection and the response to serum treatment. In general, the worse the infective process, the longer were injections of serum necessary to control the hiccup. This was especially true in cases in which there was surgical drainage after operative treatment of suppurative appendicitis, cholecystitis, and prostatitis associated with cystitis. In several cases in which the diagnosis was functional hysterical hiccup, the spasm-producing streptococcus was isolated from the nasopharynx and prompt relief followed administration of the serum.

As in epidemic encephalitis and poliomyelitis, there has been found a much higher incidence of cases of persistent hiccup—a mild form of myoclonic encephalitis—among men than among women, indicating an inherent susceptibility of men to the inciting agents of these diseases.

COMMENTS AND CONCLUSIONS

The results of previous studies have been corroborated and extended. The isolation of the spasm-producing type of streptococcus from the nasopharynx, milk supplies, and outdoor air during epidemic prevalence of hiccup and certain respiratory infections and from the air of rooms occupied by persons having persistent hiccup, and the absence of this type of streptococcus remote from hiccup, indicate that epidemic hiccup and postoperative hiccup occur in increased incidence when streptococci normally present in throats of persons and in nature generally acquire spasm-producing properties or virulence.

Persistent epidemic hiccup and persistent postoperative hiccup are considered as forms of mild myoclonic encephalitis. The inciting agent is a streptococcus *(Streptococcus singultus)* closely related to the streptococcus isolated in studies of epidemic encephalitis and the antistreptococcic serum for epidemic encephalitis is curative in persistent hiccup.

REFERENCES

1. Boyd, William: Epidemic Encephalitis: The Second Winnipeg Outbreak, Quart. J. Med. 18: 153, 1925.
2. Cadham, F. T.: Hiccup: The Winnipeg Epidemic, J. A. M. A. 84: 580, 1925.
3. Habermass, A.: Ear, Nose and Throat Symposium, Weekly Bull., St. Louis M. Soc. 19: 9, 1925.
4. Heilman, F. R., and Rosenow, E. C.: Newer Methods of Study and Treatment of Chronic Streptococcal Disease, Proc. Staff Meet., Mayo Clin. 12: 252, 1937.
5. Mayo, C. W.: Hiccup, Surg., Gynec. & Obst. 55: 700, 1932.
6. Rosenow, E. C.: Diaphragmatic Spasms in Animals Produced With a Streptococcus From Epidemic Hiccup: Preliminary Report, J. A. M. A. 76: 1745, 1921.
7. Rosenow, E. C.: The Production of Spasms of the Diaphragm in Animals With a Streptococcus From Epidemic Hiccup, J. Infect. Dis. 32: 41, 1923.

8. Rosenow, E. C.: Production of Spasms of the Diaphragm in Animals by Living Cultures, Filtrates, and the Dead Streptococcus From Epidemic Hiccup, J. Infect. Dis. 32: 72, 1923.
9. Rosenow, E. C.: Further Studies on the Etiology of Epidemic Hiccup (Singultus) and Its Relation to Encephalitis, Arch. Neurol. & Psychiat. 15: 712, 1926.
10. Rosenow, E. C.: Neuromyelo-encephalitis During and Following an Epidemic of Hiccup: Diverse Localization of Streptococci, Arch. Neurol. & Psychiat. 16: 21, 1926.
11. Rosenow, E. C.: Localization in Animals of Streptococci From Cases of Epidemic Hiccup, Encephalitis, Spasmodic Torticollis and Chorea, Arch. Neurol. & Psychiat. 19: 424, 1928.
12. Rosenow, E. C.: Isolation of Bacteria From Virus and Phage by a Serial Dilution Method, Arch. Path. 26: 70, 1938.

CIRCULATING TIME IN THE HUMAN BEING AND IN THE DOG AS AFFECTED BY FASTING AND BY MEALS*

I. M. Reingold, M.D., F. Neuwelt, M.D., and H. Necheles, M.D., Ph.D.
Chicago, Ill.

Our laboratory has been interested in the use of determinations of circulating time in the state of shock.[1] We were interested, therefore, in the normal variations of circulating time during fasting and following the ingestion of various foods. Our results obtained differ from those of another group.[7, 8]

Various reports in the literature indicate that there is a considerable change in circulation during digestion in man. According to Grollman[2] and others, a significant increase in cardiac output occurs in man after ingestion of food and even of water. Booth and associates[3] reported an elevation of skin temperature in normal subjects following a meat meal. Herrick and associates[4] did not obtain an increase of surface temperature in dogs following ingestion of foods.

In 1934 Herrick and associates[4] reported a notable increase in blood flow in the femoral, carotid, and mesenteric arteries, and in the external jugular vein of the dog following ingestion of various foods. The time of the onset and the duration of the increased flow were influenced by the character of the food taken, but the magnitude of increase was relatively the same regardless of the type of food. These results were confirmed in the human being by Burton and Murlin.[5] Abramson and Fierst,[6] working on human subjects, did not find significant changes in the rate of peripheral blood flow following a carbohydrate meal, but with a protein meal the rate of blood flow increased one and one-half to three hours after ingestion, first in the hand, and later in the forearm and leg.

In 1934 Sheard and co-workers[7] reported experiments in which the circulating time of the blood of dogs was reduced 20 to 35 per cent during digestion of a mixed meal. The lowered circulating time coincided with increases in blood flow measured simultaneously. The method used by these workers consisted in the injection of radium C into the jugular vein and the measurement

*From the Department of Gastro-Intestinal Research, Michael Reese Hospital, Chicago. Supported by the Emanuel Friend Fund.
Received for publication, June 29, 1942.

of the interval of time between the injection and the arrival of the radium below an ionization chamber located over the femoral artery. In 1936 they confirmed these results.[8]

METHODS

In the work reported here we have used the sodium cyanide method for the determination of circulating time, as first described by Loevenhart and associates[9] and as applied by Robb and Weiss to the human being.[10] The cyanide method consists of the intravenous injection of a small quantity of sodium cyanide and the measurement of the time elapsing between the injection and the first response of the chemoreceptors of the carotid sinus which stimulate respiration. In the dog 1 mg. of sodium cyanide dissolved in 0.5 c.c. of saline was injected into the saphenous vein of the hind leg, and in man 8 mg. of sodium cyanide in ⅓ c.c. of saline solution was injected into the antecubital vein. The injection was given rapidly, and two stop watches were started at the time of injection. At the height of the first deep gasp one of the stop watches was arrested. The time obtained was considered to be the circulating time of the blood between the peripheral vein and the carotid sinus. The reaction consisted of a series of rapid and deep respirations. Two stop watches were used because an occasional deep breath not followed by a series of rapid and deep respirations might yield a false end point, which could be corrected by the use of the second stop watch when the series of rapid and deep respirations began.

The human subjects and the animals were starved for at least twelve hours. The experiments on the dogs were performed on two healthy animals and those on the human subjects, on workers in the laboratory, and on such patients of the wards who had undergone simple operations and were ready to be dismissed, and on such patients in whom the circulatory system did not seem to be affected by the disease. The ages of the subjects varied between 16 and 79 years, with an average of 49 years. Most of the human subjects were lying in bed during the experiments, and a few were up and around. The dogs were in a stall during the entire experiment.

A meal was administered after one or more control determinations of the circulating time had been performed, and determinations were repeated at various intervals following ingestion of the meal. One group of subjects was followed over a prolonged period of time without administration of food. The meals administered consisted of protein foods (steaks), or of a regular full hospital diet, or of carbohydrate and fat foods. The amounts eaten were about those of a good average luncheon and varied somewhat according to the appetite of the subject. The dogs were fed one pound of a commercial dog food containing meat, oats, and vegetable, mixed with water.

Twenty-nine experiments were performed on 21 different subjects: 17 males and 4 females. Twelve experiments were performed on 12 different fasting subjects and are recorded on the upper part of Chart 1. Six experiments were performed on two dogs.

RESULTS

A. In Human Subjects.—Fasting circulating times varied between 12 and 22 seconds, with an average of 17.2 seconds (34 tests). Controls before meals varied between 12 and 29 seconds, with an average of 17.6 seconds (31 tests).

Circulating times following all meals varied between 10 and 26.6 seconds, with an average of 17.5 seconds (48 tests). Analysis of the data did not reveal significant differences in the changes of circulating time following the various meals. In order to illustrate the wide variations of circulating time during fasting and following meals, we have expressed all data as per cent changes from the first determination in the fasting subjects and from the last determination before the meal in the postprandial subjects. We have chosen this method of presentation of our data because the variations observed during the fasting and the postprandial states seemed to be due to cyclic changes.

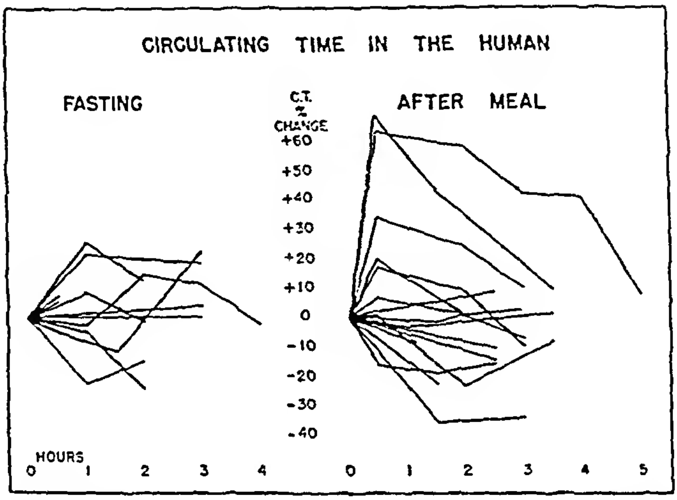


Chart 1.—Circulating time in the *fasting* human subject in per cent changes from first determination. Circulating time *after meal* in per cent changes from last determination before meal.

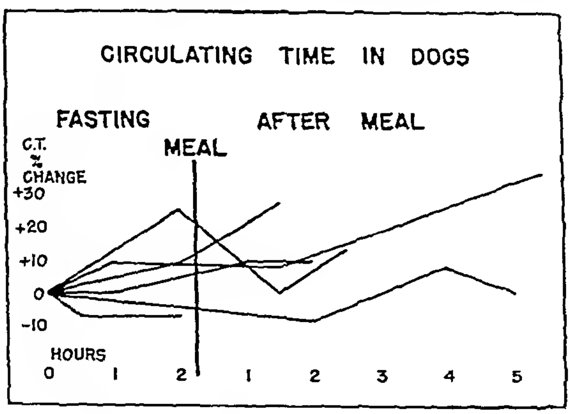


Chart 2.—All circulating times in per cent changes from first determination made.

In most of the fasting subjects variations between −25 and +25 per cent from the control reading were obtained, while in only a few of the fasting subjects circulating time did not vary appreciably during the periods of observation of thirty minutes to four hours.

Seventeen experiments following ingestion of food are recorded on the right side of Chart 1. Circulating time varied between a diminution of 36 per cent and an increase of 67 per cent of the control values. The spread of the

data is greater than that seen during fasting, but this is due to a greater variation in circulating time in *only 2 of these 17 experiments.*

B. In the Dog.—Values of circulating time before the meal varied between eight and fourteen seconds, with an average of twelve seconds. (12 tests), and after the meal they varied between eight and fifteen seconds, with an average of 12.2 seconds (11 tests). Chart 2 illustrates that essentially the same results were obtained in the dog as in the human being. The circulating time was not constant during periods of two hours of fasting and did not show uniform changes in any direction during periods of one and one-half to five and one-half hours following ingestion of the protein meal.

DISCUSSION

Our results indicate that circulating time varies considerably during fasting as well as following ingestion of various foods. These variations were not uniform in direction, and prolongation as well as shortening of circulating time might occur. From the distribution (spread) of data, as well as from the algebraical averages we assume, therefore, that the changes of circulating time following a meal are not any more significant than are those during fasting. The variations of the individual curves during fasting as well as following meals may be due to cyclic variations in circulating time, rather than to specific causes, such as anxiety, the *postabsorptive state*, etc. If it can be proved that this is due to cyclic variations in circulating time, it will make the simple use of this method uncertain for a number of clinical determinations. The use of the method is not without value in selected conditions, however, as, for example, in the state of shock, where we have described changes far beyond those observed in normal dogs.[1]

We cannot explain the difference between our results and those of the Mayo group.[7, 8] Of course, the two methods are entirely different, and it is possible that they are affected differently during the absorptive state. The sensitivity of the chemoreceptors of the carotid sinus is so great that differences in their response to cyanide constitute only a small fraction of the total circulating time,[1] and we feel that a meal could not affect these receptors so much as to change the total circulating time to any extent. There may be other factors at work, however, such as changes of the circulating volume or of the hematocrit-plasma ratio during the absorptive state, or differences in the carriers (red blood cells or plasma) of the different agents employed, which may affect both methods differently. Besides, it was not apparent from the report of the Mayo group whether prolonged control periods during starvation had been instituted and had been compared with the variations of circulating time following meals.[7] In another paper, however, this group reported considerable variations of the circulating times in individual fasting dogs.[11]

The reported constant differences between the cyanide method and arm-to-tongue methods[12] would not seem to affect our findings.

We feel that our results are also of importance because the type of method employed in our study for determination of circulating time belongs to those in common use.

SUMMARY

The circulating time was determined in a group of human subjects and in dogs. The sodium cyanide method of Loevenhart and associates was employed. During the fasting condition considerable variations of circulating time were found in the human being and in the dog, and following meals the same or somewhat greater variations in circulating time were obtained. Our results indicate no significant or uniform changes in circulating time in any one direction in either the fasting or the postprandial state. The nature of the meal did not seem to determine changes in circulating time. The significance of our findings and the discrepancy of our results from those of other workers are discussed.

REFERENCES

1. Olson, W. H., Gutmann, H., Levinson, S. O., and Necheles, H.: Circulating Time in Shock, War Med. 1: 830, 1941.
2. Grollman, A.: The Cardiac Output of Man in Health and Disease, Springfield, Ill., 1932, Charles C Thomas, p. 95.
3. Booth, G., Strang, J. M., and Evans, F.: Skin Temperature Changes Following Food Digestion, J. Clin. Investigation 13: 686, 1934.
4. Herrick, J. F., Essex, H. E., Mann, F. C., and Baldes, E. J.: The Effect of Digestion on the Blood Flow in Certain Blood Vessels of the Dog, Am. J. Physiol. 108: 621, 1934.
5. Burton, A. C., and Murlin, J. R.: Human Calorimetry. III. Temperature Distribution, Blood Flow and Heat Storage in Basal Condition and After Ingestion of Food, J. Nutrition 9: 281, 1935.
6. Abramson, D. I., and Fierst, S. M.: Peripheral Vascular Responses in Man During Digestion, Am. J. Physiol. 133: 686, 1941.
7. Sheard, C., McCracken, E. C., and Essex, H. E.: The Circulation Time of the Blood in Dogs Before and During the Digestion of Food, Proc. Am. Physiol. Soc., 46th Annual Meeting, March, p. 96, 1934.
8. Sheard, C., McCracken, E. C., and Essex, H. E.: The Circulation Time of the Blood in Dogs as Measured by Ionization Methods, Effect of Various Physicophysiologic Agents and of Drugs, Proc. Am. Physiol. Soc., 48th Annual Meeting, March, p. 142, 1936.
9. Loevenhart, A. S., Schlomovitz, B. H., and Seybold, E. G.: The Determination of the Circulation Time in Rabbits and Dogs and Its Relation to the Reaction Time of the Respiration to Sodium Cyanide, J. Pharmacol. & Exper. Therap. 29: 221, 1922.
10. Robb, G. P., and Weiss, S.: A Method for the Measurement of the Pulmonary and Peripheral Venous Blood Flow in Man, Am. Heart J. 8: 650, 1933.
11. McCracken, E. C., Essex, H. E., and Sheard, C.: The Circulation Time of the Blood of Dogs, Determined by Ionization (Geiger Counter) Methods. I. The Effect of Physicophysiological Agents and of Drugs, Am. Heart J. 14: 51, 1937.
12. Kvale, W. F., and Allen, E. V.: The Rate of the Circulation in the Arteries and Veins of Man. IV. An Error in the Sodium Cyanide Method of Determining Speed of Venous Blood Flow, Am. Heart J. 18: 557, 1939.

RECURRENT ATTACKS OF PNEUMOCOCCIC PNEUMONIA TREATED WITH SULFONAMIDES*

LEON SCHWARTZ, M.D., HARRISON F. FLIPPIN, M.D., AND
JEFFERSON H. CLARK, M.D., PHILADELPHIA, PA.

RECURRENCE is more commonly seen in pneumococcic pneumonia than in any other acute infectious disease.[1] Likewise, the attack rate of pneumococcic pneumonia is higher in those persons who have had previous attacks of the disease.[2] The reported incidence of one or more previous attacks varies greatly throughout the world, although most American authorities have noted recurrences in approximately 15 per cent of cases. Furthermore, it is the opinion of many writers[3, 4] that the number of previous attacks of pneumococcic pneumonia does not influence the prognosis of the disease. Within the past four years the treatment of pneumococcic pneumonia has been revolutionized as a result of the advent of the sulfonamide compounds. It is too early to predict whether the widespread use of these drugs will have any effect upon the above-mentioned facts concerning recurrent pneumococcic pneumonia, since it will be necessary to accumulate extensive data from different sources over a period of years before an evaluation can be attempted. However, of equal interest is whether these chemotherapeutic agents are as effective when employed for repeated attacks of pneumococcic pneumonia and whether the patients tolerate the sulfonamide drugs as well during subsequent administrations. The purpose of this study is to present the comparative therapeutic effectiveness and toxicity of the sulfonamides in recurrent pneumococcic pneumonia.

During the past three years we have observed in a series of 1,400 adult pneumonias 24 patients who received sulfapyridine, sulfathiazole, or sulfadiazine on two or more occasions for pneumococcic pneumonia. In every instance the patient was discharged from the hospital fully recovered from pneumonia; and, in order to eliminate possible cases of relapse, we have excluded those readmitted within four weeks following discharge. The diagnosis of pneumonia was established in every case, and a pneumococcus was recovered from the sputum or blood in all patients, although a specific pneumococcus type was not always obtained.

As indicated in Table I, there was a total of 49 hospital admissions in this group of 24 patients. The time interval between admissions ranged from four to ninety-two weeks, with an average of thirty-seven weeks. The patients' ages varied from 14 to 74 years, with 58.3 per cent of the cases being over 40 years of age. Typable pneumococci were found in the sputum or blood stream in 35

*From the Committee for the Study of Pneumonia, Philadelphia General Hospital. Aided by a grant from the American Philosophical Society.
Received for publication, July 8, 1942.

TABLE I

SIGNIFICANT DATA ON 24 CASES OF RECURRENT PNEUMOCOCCIC PNEUMONIA TREATED WITH SULFONAMIDES

		FIRST ATTACK					INTERVAL BETWEEN ATTACKS (WEEKS)	SUBSEQUENT ATTACKS				
NO.	AGE YR.	TYPE	LOBES INVOLVED	TREATMENT	RESULTS	TOXIC EFFECTS		TYPE	LOBES INVOLVED	TREATMENT	RESULTS	TOXIC EFFECTS
1	68	VIII	RM, RL	S-P*	Good	Vomiting	32	III	RL	S-P	Good	Vomiting
2	57	VI	RL	S-P	Good		13	V	RL	S-P	Good	
3	37	I	LL	S-P	Good	Vomiting	4	–	RL, LL	S-P	Good	Vomiting
4	74	III	RL, LL	Serum S-P	Good	Vomiting, microhematuria	20	III	RL	S-P	Good	Vomiting, microhematuria
4a†							8	III^b	RL, LL	S-P	Died	Vomiting, microhematuria
5	21	–	RU, RM	S-P	Good		4	XIV	RU	S-P	Good	
6	42	VII	LL	S-P	Good		37	VIII	RL	S-P	Good	
7	14	IV	LL	S-P	Good		56	–	LL	S-P	Good	
8	40	–	RL	S-P	Fair		33	–	RL, LL	S-P	Fair	
9	68	VII	RL	S-P	Good	Vomiting	62	VII	RL	S-T	Good	Vomiting, microhematuria
10	66	V^b	LU	S-P	Fair		22	I	RM	S-T	Good	
11	49	VIII	LL	S-P	Fair		42	–	LL	S-T	Good	
12	32	III^b	RL, LL	Serum S-P	Good	Vomiting	58	VIII	RL, RM	S-T	Good	
13	27	III	LU	S-P	Good	Vomiting	40	VII	RL	S-T	Good	
14	25	VII	RL, LL	S-P	Fair	Vomiting	26	–	LL	S-T	Good	
15	55	–	LL	Serum S-P	Good	Vomiting	18	–	LL	S-T	Good	Vomiting
16	37	XXIV^b	LL	S-P	Good		92	XIX	LU	S-D	Good	
17	60	–	LU	S-P	Good	Microhematuria	84	XXIX	LU, LL	S-D	Good	
18	27	–	RM	S-T	Good		4	XIV	LL	S-T	Good	
19	53	VIII	LU	S-T	Fair	Vomiting	42	VIII	RL	S-T	Fair	
20	66	V^b	LU	S-T	Fair	Microhematuria	22	I	RM	S-P	Good	
21	44	III	LL	S-T	Good		39	III	RL, LL	S-P Serum	Died	Vomiting
22	48	VII	LL	S-T	Good		52	III	RL	S-D	Good	
23	26	–	LU	S-T	Good		49	I^b	RL	S-D	Good	
24	57	–	RL	S-T	Fair	Vomiting, microhematuria	53	–	LL	S-D	Fair	

*S-P = Sulfapyridine. S-D = Sulfadiazine. b = Positive blood culture.
S-T = Sulfathiazole. †4a = Third admission.

instances. The same pneumococcus type was the causative agent for subsequent attacks in only 4 patients. Positive blood cultures were obtained in 4 patients on the initial admission and in 2 patients on subsequent admission to the hospital. In 14 patients the same lobe of the lung was involved during each attack of pneumonia, and in 9 of this subgroup an additional lobe was involved. There was no appreciable difference in the total number of lobes involved during the initial and subsequent attacks.

Sulfapyridine was employed in 17 patients during their first admission, and of this number 8 received the same drug on nine subsequent admissions. The remainder of this subgroup received sulfathiazole (7 patients) or sulfadiazine (2 patients). Seven patients were given sulfathiazole on their first admission, and of these 2 received sulfapyridine, 2 sulfathiazole, and 3 sulfadiazine on other admissions. Good therapeutic response to treatment with these drugs was determined by a critical fall in temperature within forty-eight hours, a return to normal temperature within forty-eight to seventy-two hours, and by the findings on physical examination. On this basis we have designated the results of treatment as good, fair, or died. There were 2 deaths (8.0 per cent mortality) in this group of 25 subsequent admissions, both occurring in patients with type III infection. This mortality rate compares favorably to that in a larger series of cases of pneumococcic pneumonia treated with sulfonamides reported from this hospital.[5] Of the 22 patients surviving both attacks of the disease, the results of treatment were considered good in 15 on their first admission and in 20 during subsequent courses of therapy with these drugs. This difference in therapeutic response on the different admissions is of no statistical significance.

The incidence of severe toxic reactions due to the drugs is very small in this series. Vomiting and microscopic hematuria constituted the only toxic manifestations worthy of mention. In comparing these toxic effects it should be pointed out that the selection of drug undoubtedly influenced the incidence of toxicity in these cases. However, it is of interest to note that none of the more serious toxic reactions developed on subsequent use of the same drug or of other members of this group of drugs. Not included in this series of cases is a patient recently treated by one of us (H. F. F.) for pneumonia with sulfadiazine who showed no apparent ill effects from the drug; this patient had on a previous occasion developed a severe skin reaction as a result of sulfathiazole medication.

In view of the data presented in this report it would appear that the use of the sulfonamides in a previous attack of pneumococcic pneumonia does not alter the response to the same drug or to similar drugs when used in subsequent attacks of the disease. Also, the incidence of toxic reactions is no greater when these drugs are employed for repeated attacks of pneumococcic pneumonia. Further study of similar data on a larger series of cases receiving the same drug for two or more attacks of pneumococcic pneumonia is desirable.

SUMMARY

1. Twenty-four adult patients have been given sulfonamide therapy on two or more occasions for recurrent pneumococcic pneumonia.

2. The drugs seemed just as effective when administered for subsequent attacks of the disease as during their initial use.

3. There is no evidence to suggest that the repeated use of the sulfonamides for recurrent pneumococcic pneumonia increases the incidence or severity of drug toxicity.

REFERENCES

1. Cecil, R. L.: A Textbook of Medicine, ed. 5, Philadelphia and London, 1940, W. B. Saunders Co., page 136.
2. Heffron, R.: Pneumonia With Special Reference to Pneumococcus Lobar Pneumonia, The Commonwealth Fund, New York, 1939, page 539.
3. Cole, R. I.: Acute Lobar Pneumonia, Nelson's Loose Leaf Living Medicine, New York, 1920-1928, Thomas Nelson and Sons, vol. 1, page 203.
4. Warr, O. S., and Alperin, J.: Lobar Pneumonia, A Report of 2,039 Cases, Ann. Int. Med. 6: 1474, 1933.
5. Flippin, H. F., Schwartz, L., and Clark, J. H.: Factors Influencing the Fatality Rate of Pneumococcic Pneumonia Treated With Sulfonamide Compounds, Ann. Int. Med. 14: 1969, 1941.

ERYTHROCYTE SEDIMENTATION RATE DETERMINATIONS ON NORMAL YOUTHS

C. Frederic Roche, M.D., F.A.C.P., Donald G. Stannus, M.D., and Emil M. Isberg, M.D., Miami Beach, Fla.

The erythrocyte sedimentation rate is an accepted laboratory measure of pathologic activity, and the range of normal values for the various techniques has been determined by numerous investigators over the country. Rourke and Ernstene found the normal range of "corrected sedimentation index" to be 0.08 to 0.35 mm. per minute, based on the results of 92 measurements done on 45 different healthy persons.

In order to ascertain what is the range of normal in this community, determinations were done on 100 normal youths, all students at the junior high school and high school here. A careful history and physical examination were done on each subject, and only those found to be in good health were included in the series. Any history of recent infection, common cold, tonsillitis, dental infection, sinusitis, etc., excluded the potential subject. Of the 100 healthy youths in the series, 60 were males and 40 were females. Their ages ranged from 11 to 17 years.

The temperature of each subject was recorded, and all blood specimens were obtained at least two hours after the previous meal. Extreme care was taken in order that blood be withdrawn from the vein with as little stasis as possible. Any apprehension or excitement on the part of the subject at the time the specimen was withdrawn was recorded. The date on which the last menstrual flow ceased was noted for each female subject.

The Rourke-Ernstene corrected sedimentation technique was used exclusively. Each determination was made within two hours after the blood speci-

Received for publication, July 6, 1942.

men was obtained. Heparin was used as the anticoagulant. Every sedimentation tube was handled with a minimum amount of manipulation. Precautions were taken to see that the sedimentation tubes were in absolute vertical position. The temperature and humidity of the laboratories and the barometric pressure were recorded. Sixty-two determinations were done in the St. Francis Hospital laboratory, which is not air-conditioned, and the remaining 38 tests were done in an air-conditioned laboratory.

The corrected sedimentation rates of the 100 normal youths ranged from 0.09 mm. per minute to 2.0 mm. per minute. The mean rate was 0.46 mm. per minute. Seventy-five per cent of all the determinations were below 0.65 mm. per minute. Rourke and Ernstene[1] placed the value of 0.35 mm. per minute as the upper limit of normal. Yet 53 per cent of all the readings in this series fell in the range between 0.35 and 0.65 mm. per minute. The mean rate for girls was 0.47 mm. per minute, and for boys it was 0.46 mm. per minute.

We could not determine the reason that the sedimentation phenomenon should be more rapid in a subtropical climate. Numerous investigators have shown that low temperatures retard and high temperatures accelerate the rate of sedimentation of erythrocytes. The barometric pressure varied from 760 mm. to 770 mm.

Forty-four volumes per cent was found to be the mean hematocrit value for both males and females in our series. Rourke and Ernstene have set 45 volumes per cent as the mean value for correcting the sedimentation rate. Forty-nine volumes per cent was the highest value for females in the series, while 11 of 60 males had hematocrit values of 50 to 52 volumes per cent.

CONCLUSIONS

1. The erythrocyte sedimentation rates of 100 normal Miami Beach youths were determined according to the Rourke-Ernstene technique.

2. The rates of 75 per cent of the series ranged from 0.09 to 0.65 mm. per minute, and 53 per cent of the series ranged from 0.35 to 0.65 mm. per minute.

3. We propose that the upper limit of normal for the Rourke-Ernstene corrected sedimentation index for South Florida be extended to 0.65 mm. fall per minute.

4. Since it has been proved by other workers that the various methods for determining erythrocyte sedimentation rate are reasonably the same, we suggest that the range of normal for other techniques also be extended for this locality.

REFERENCE

Rourke, M. D., and Ernstene, A. C.: Method for Correcting Erythrocyte Sedimentation Rate for Variations in Cell Volume Percentage of Blood, J. Clin. Investigation 8: 545, 1930.

541 LINCOLN ROAD

PAPILLARY LYMPHOID CYSTADENOMA*

Report of Four Cases

Hyman Peck, M.D., Hines, Ill.

AMONG tumors of the salivary glands one of the rarest is papillary lymphoid cystadenoma. Other terms by which it has been described include papillary adenolymphoma, papillary cystadenoma lymph glands, papillary cystadenoma lymphomatosum, branchiogenic cystadenolymphoma, branchiogenic adenoma, branchioma, and onkocytoma. Papillary lymphoid cystadenoma is probably the most descriptive name for the tumor.

The tumors were first described by Albrecht and Arzt (1910), Glass (1912), Ssobolew (1912), and Feldman (1916). In 1935 Carmichael, Davie, and Stewart reviewed the literature and found 26 cases reported up to that time in twenty-one papers. They described 8 new cases. Subsequently, five more cases appeared in the American literature,[6, 8, 9, 11] and seven in foreign language publications.[10-16]

The genesis of these tumors has been variously ascribed to heterotopic salivary gland tissue in lymph nodes,[3, 4] heterotopic pharyngeal epithelium,[17] and to branchiogenic rests.[18] The great majority of the tumors are first noticed in the fifth or sixth decade, and they are found only in close relation to the parotid and submaxillary glands and at the angle of the jaw. No case has been noted in association with the sublingual glands. They are six or seven times more common in males than in females. Only two of the cases have been stated to be malignant. One tumor reported by Oden[6] showed enough active proliferation and loss of polarity of the cells to be possibly a transition stage between benignity and malignancy. Only one case was reported in which there was a local recurrence and this appeared in three years.

In the surgical biopsies of Hines Hospital during the past two years five cases of papillary lymphoid cystadenoma were found. One of these has been reported by Hines.[11] All of them were benign.

CASE REPORTS

Case 1.—The patient, W. S. D., was a white male, aged 51 years. One and a half years before admission to the hospital he noticed in the preauricular area on the right a small lump which had grown progressively larger. On physical examination this mass was small and movable.

At operation the tumor was enucleated from under the capsule of the parotid gland. Another smaller nodule was then found adjacent to the one previously removed, and it was also removed. The tumors measured 2 by 1 by 1 cm. and 1.5 by 1 by 0.5 cm.

Case 2.—Three and a half years before admission to the hospital a white male, A. J. K., aged 49 years, observed in the right parotid region a growth which gradually

*From the Clinical Laboratory Center, Veterans' Administration, Hines, Ill.

Published under R & P 6227.
Received for publication, July 13, 1942.

increased in size. No discomfort or pain was associated with the lesion. He was operated on at another hospital, and a partial excision was done. The biopsy diagnosis was made there of adenoma of the parotid with malignant change. Upon admission to the hospital for further therapy one month later a soft 2 cm. mass was found in the right parotid area. The tumor was raised 1.5 cm. above the skin. Sialogram revealed filling of the ducts of the parotid gland which was compressed by the external tumor.

At operation a nodule was found in the substance of the parotid gland. The tumor was completely encapsulated and easily enucleated. It measured 4.5 by 2.5 cm.

Case 3.—Patient, R. C. B., a white male, aged 48 years, noticed a small lump behind the left ear, five months before admission to the hospital. The mass was about the size of a pecan and had grown steadily larger. The patient experienced no pain and no increase in salivation.

Physical examination revealed a 5 by 4 cm. mass in the left parotid region. The overlying tissues were freely movable, but the tumor appeared to be somewhat fixed to the underlying structures. No adenopathy was present.

At operation the tumor was found to be within the parotid gland substance but well encapsulated and easily enucleated. It measured 5.3 by 3.5 by 2 cm., felt cystic, and had small satellite nodules at the periphery.

Case 4.—The patient, G. F. J., a white male, aged 46 years, noticed a mass behind the angle of the left jaw three years before admission to the hospital. This became progressively larger. The nodule was asymptomatic.

Physical examination revealed a tumor the size of a walnut behind the angle of the left jaw. The skin was freely movable over it. The mass was slightly movable but was apparently attached to, and was part of, the parotid gland underneath.

At operation this mass was found in the lower pole of the parotid gland. It was encapsulated and was easily enucleated. It measured 4 by 3 by 2 cm.

The preoperative duration in our cases was eighteen months, forty-two months, five months, and thirty-six months, respectively, and averaged twenty-five months. The tumors were in the substance of the parotid gland in three cases and just under the capsule in the first case. None of the masses were painful or tender. The tumor was not attached to the skin in any of the cases. The right and left parotid areas were equally affected in frequency. The preoperative diagnosis in three cases was mixed tumor of the parotid gland. In our second case the preoperative diagnosis was adenoma of the parotid with malignant change. All the tumors were well encapsulated and easily removed.

The capsule of the tumors was thin, fibrous, and had small vessels on its surface. The color varied from a reddish brown to a reddish gray. There was a small nodular character to the surface. The tumors were cut with ease and the sectioned surface was finely granular and showed small cysts filled with serous or colloid material which upon formalin fixation became an opalescent pearly gray. The specimen in our first case, however, showed no cysts grossly.

Histologically the tumors were cystic with papillary structures. They were composed of a double layer of columnar epithelial cells which formed tubular alveoli or clefts, and cystic spaces with slender and branching papillary projections. The cells were pale and under low magnification the cell outlines were difficult to make out. Goblet cells were very prominent and numerous in the fourth case and appeared occasionally or were absent in the other cases. The basal cells were smaller, irregular, and lay close to the basement membrane. In places the cells were multilayered. The nuclei were round to oval and had a delicate chromatin network. Some of the nuclei were indented. The nuclei had

Fig. 1.—Gross appearance of papillary lymphoid cystadenoma in fourth case.

Fig. 2.—Structure of papillary lymphoid cystadenoma in fourth case, showing double layer of lining epithelium, papillary structure, and lymphoid follicles in stroma. Magnification ×150.

one or two nucleoli. The epithelium rested upon a thin membrane which seemed to fuse with the delicate reticulum of the stroma in the center of the papillae. The stroma was infiltrated by many lymphocytes, with the formation of lymph follicles having large reaction centers. The cells were not ciliated. However, adherent masses of secretion gave a false appearance of cilia under low magnification.

The material in the cysts consisted of granular precipitate with desquamated epithelium, lymphocytes, phagocytes, fat, and cholesterol.

SUMMARY

1. Of 6,239 surgical biopsies in Hines Hospital four cases of papillary lymphoid cystadenoma were encountered.

2. All four cases showed considerable consistency in clinical and gross and histological characters.

3. The clinical features were a painless and otherwise asymptomatic slow-growing mass in close association with the parotid gland. The overlying skin was movable and no ulceration occurred. The tumors were completely encapsulated and easily enucleated and they did not recur.

4. The gross appearance was characterized by a somewhat nodular capsule and a finely granular cut surface on which cysts could usually be easily seen.

5. The histology was characteristic and essentially consisted of papillary structures with a double layer of cells lining cystic spaces. The core of the papillae was made up of a reticulum infiltrated with lymphocytes forming follicles having large reaction centers.

REFERENCES

1. Albrecht, H., and Arzt, L.: Beitrage zur Frage der Gewebsverirrung. I. Papillare Cystadenome in Lymphdrusen, Frankfurt. Ztschr. f. Path. 4: 47, 1910.
2. Glass, E.: Über ein branchiogenes papillares Cystadeno-Lymphom der Regio parotides. Frankfurt. Ztschr. f. Path. 9: 335, 1912.
3. Hamperl, H.: Onkocyten und Geschwulste der Speicheldrusen, Arch. f. Anat. 282: 724, 1931.
4. Jaffe, R. H.: Adenolymphoma (Onkocytoma) of the Parotid Gland, Am. J. Cancer 16: 1415, 1932.
5. Carmichael, R., Davie, T. B., and Stewart, M. J.: Adenolymphoma of the Salivary Glands, J. Path. & Bact. 40: 601, 1935.
6. Oden, C.: Papillary Cystadenoma Lymphomatosum of the Parotid Gland, Am. J. Surg. 30: 57, 1935.
7. Hall, E. M.: Adenolymphoma of the Parotid Gland, Arch. Path. 19: 756, 1935.
8. Harris, P. N.: Adenocystoma Lymphomatosum of Salivary Glands, Am. J. Path. 13: 81, 1937.
9. Freshman, A. W., and Kurland, S. K.: Am. J. Clin. Path. 8: 422, 1938.
10. Caeiro, J. A., and Monserrat, J. L.: An. Inst. modelo de clin. méd. 17: 569, 1936.
11. Hines, R. E.: Papillary Cystadenoma Lymphomatosum, M. Bull. Vet. Admin. 18: 210, 1941.
12. Tello, J. F., and Herrera, J. M.: Papillary Cystadenolymphoma of Neck, Trav. du. lab. de recherches biol. de l'Univ. de Madrid 29: 157, 1934.
13. Kreudenstein, S. von: Papillary Cystadenoma With Lymphatic Interstitial Tissue in Parotid Gland, Arch. f. klin. Chir. 182: 828, 1935.
14. Scandurra, S.: Papillary Cystadenolymphoma of Neck, Arch. ed atti d. Soc. ital. di chir. 40: 704, 1934.
15. Matsushima, K.: Papillary Cystadenolymphoma of Parotid Gland, Ztschr. d. japan. chirurg. Gesellsch. 36: 5, 1935.
16. Venzoni, M.: Adenolymphoma of Salivary Glands, Arch. ital. di chir. 58: 465, 1940.
17. Warthin, A. S.: Papillary Cystadenoma Lymphomatosum, J. Cancer Research 13: 116, 1929.
18. Feldman, I.: Adenoma Branchiogenes, Centralbl. f. allg. Path. u. path. Anat. 27: 25, 1916.

THE SIGNIFICANCE OF THE ELECTROCARDIOGRAM WITH PROMINENT S WAVES IN LEADS I, II, AND III*

M. WILBURNE, M.D., AND R. LANGENDORF, M.D.
CHICAGO, ILL.

ASHMAN and Hidden[1] have recently directed attention to an electrocardiographic pattern exhibiting prominent S waves in Leads I, II, and III. Eight of their 29 cases revealed no demonstrable heart disease, and 7 of their 8 cases showed no electrocardiographic changes other than the deep S waves. In all 8 cases the patients had a vertical or normally placed heart. Only one subsequent reference (Ashman and Hull[2]) to this type of electrocardiogram has appeared in the literature so far as we know.

It seemed desirable to investigate this pattern further in order to evaluate its frequency and significance. A total of 1,850 consecutive electrocardiograms from the Heart Station of Michael Reese Hospital were reviewed. Prominent S waves (defined below) in Leads I, II, and III were present in 84 of these cases (4.5 per cent). The ages represented in the 84 electrocardiograms ranged from 4 to 77 years inclusive. Fifty-nine persons were males and 25 were females.

TABLE I

CORRELATION OF CLINICAL FINDINGS WITH ELECTROCARDIOGRAMS EXHIBITING PROMINENT S WAVES IN LEADS I, II, AND III

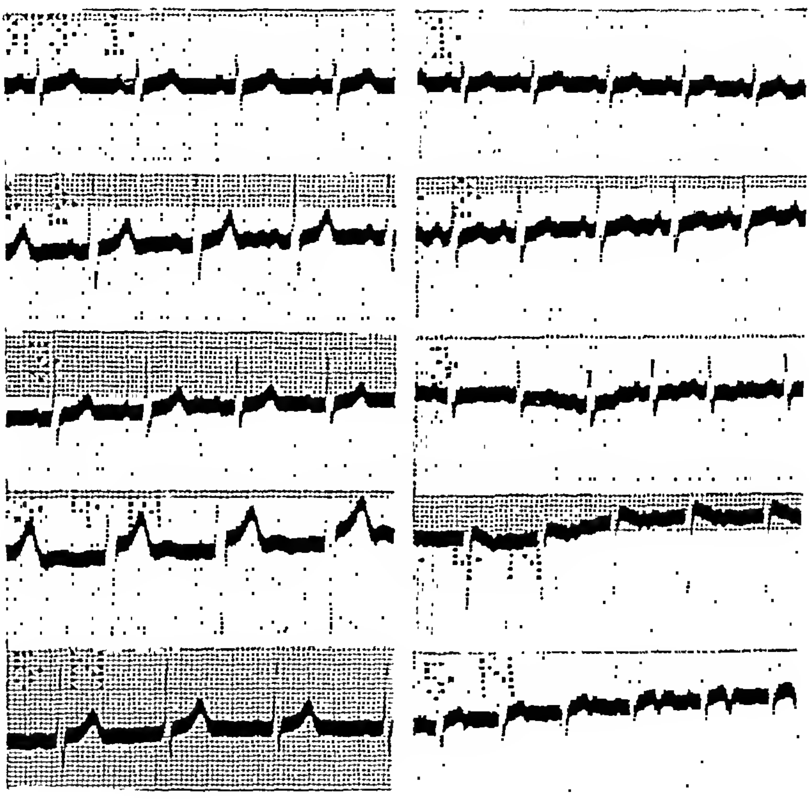
CLINICAL FINDINGS	TOTAL NO. OF CASES 84	ELECTROCARDIOGRAM		
		NORMAL* (GROUP A) 35	QUESTIONABLE (GROUP B) 8	ABNORMAL (GROUP C) 41
No demonstrable heart disease	28	19	4	5
Questionable heart disease	10	3	1	6
Definite heart disease	46	13	3	30

*See criteria in text.

The electrocardiograms were divisible into three groups: Group A, in which no additional deviations from the currently recognized normal electrocardiogram were found—35 cases; group B, in which the electrocardiograms showed changes which were considered questionably abnormal—8 cases; and group C, in which the electrocardiograms revealed definite abnormalities—41 cases. The relationship of this pattern to the presence of heart disease in these three groups is summarized in Table I, and their distribution by age groups is shown in Table II.

*From the Cardiovascular Department, Michael Reese Hospital, Chicago.
Aided by the A. D. Nast and Emil and Fanny Wedeles Funds for Cardiovascular Research.
Received for publication, July 13, 1942.

The electrocardiograms considered to be of the otherwise normal S type (Fig. 1) were those exhibiting (1) prominent S waves* in Leads I, II, and III, (2) absence of a prominent Q wave,† and (3) absence of other electrocardiographic changes. The criteria employed to differentiate the otherwise normal S type from the abnormal patterns were: (1) QRS of normal duration; (2) QRS_1 mainly upright, or small and equiphasic; (3) QRS_2 mainly upright; (4) S_2 deeper than S_3; (5) normal S-T-T complex in all limb leads; (6) CF_2 and CF_4 normal,‡ and (7) absence of low "voltage." In all the otherwise normal S type of records QRS in the limb leads was largest in Lead II. The 35 cases in group A fulfilled the above requirements. Those in groups B and C did not.



A. *B.*

Fig. 1.—Five lead electrocardiograms of the otherwise normal S type showing Leads I, II, III, CF_2, and CF_4. Record *A* is that of a normal adult and record *B* of a normal child. Note the prominent S waves in Leads I, II, and III, and the absence of other deviations from the normal. The T-wave changes in Leads CF_2 and CF_4 in *B* are normal in a child. QRS in CF_2 is diphasic but the upright phase is not clear in the reproduction.

Of the 13 cases in group A in which heart disease was known to be present, there were 5 patients with clinically quiescent rheumatic heart disease and suspected mitral insufficiency, 4 with angina pectoris, 2 with chronic cor pulmonale, one child with a first attack of acute rheumatic fever, and one child with postscarlatinal myocarditis.

---

*Final inverted phase which is 25 per cent or more of the upright phase of the QRS complex.

†Initial inverted phase which is 25 per cent or more of the upright phase of the QRS complex.

‡Three of the 84 electrocardiograms had no chest leads; two records had only one chest lead (CF_2); the remaining 79 had two chest leads (CF_2 and CF_4).

The abnormal electrocardiograms exhibiting S waves in the limb leads, represented by the 41 cases of group C, were divisible into 5 major patterns: (1) left ventricular preponderance, (2) right ventricular preponderance, (3) combined right and left ventricular strains,[3-5] (4) myocardial infarction, and (5) nonspecific patterns. In Table III is presented a summary of the various types of patterns encountered in the abnormal electrocardiogram of group C. The most frequent abnormalities encountered in the nonspecific patterns were S-T depressions in the limb leads, splintering and inversion of the QRS complex in Leads CF_2 and CF_4, inversion of the T wave in CF_2 and CF_4, and low "voltage."

TABLE II

AGES REPRESENTED IN THE 84 ELECTROCARDIOGRAMS EXHIBITING PROMINENT S WAVES IN LEADS I, II, AND III

AGES (YR.)	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80
Number of cases	8	14	8	9	13	16	13	3
No demonstrable heart disease	2	8	6	6	4	1	1	0
Questionable heart disease	1	1	1	2	1	2	2	0
Definite heart disease	5	5	1	1	8	13	10	3

TABLE III

CLINICAL STATUS AND ABNORMAL ELECTROCARDIOGRAPHIC PATTERNS EXHIBITING PROMINENT S WAVES IN LEADS I, II, AND III

CLINICAL STATUS	TOTAL NO. OF CASES	ELECTROCARDIOGRAPHIC PATTERNS				
		LEFT VENTRICULAR PREPONDERANCE	RIGHT VENTRICULAR PREPONDERANCE	COMBINED RIGHT AND LEFT VENTRICULAR STRAIN	MYOCARDIAL INFARCTION	NONSPECIFIC ABNORMALITIES
Total	41	10	4	2	4	21
No demonstrable heart disease	5	2	2			1
Questionable heart disease	6	1				5
Definite heart disease	30	7	2	2	4	15

DISCUSSION

Records with deep S waves, in Leads I, II, and III were thus encountered in 4.5 per cent (84 cases) of the 1,850 consecutive electrocardiograms reviewed; 2.2 per cent (41 cases) revealed electrocardiographic abnormalities other than the S-type pattern, indicating a definitely abnormal record; and 0.5 per cent (8 cases) showed borderline electrocardiographic variations. In 1.9 per cent (35 cases) no additional deviations from the normal were noted. This last group was labelled the otherwise normal S type. In 19 cases, or more than half of this group, this pattern occurred in patients with no demonstrable heart disease. The probabilities are that the percentage of normal hearts in this group would be much greater in a random sample of the population than in the one selected for this study, since the percentage of normal hearts included would have been much greater.

This otherwise normal S type of electrocardiogram (in which prominent S waves are present in the three limb leads without other deviations from the currently recognized normal electrocardiogram[6]) is thus encountered in ap-

proximately 2 per cent of records in electrocardiographic files. The question arises as to whether the otherwise normal S type of electrocardiogram should be regarded as a normal variant frequently observed in cardiac disease, or as an abnormal pattern often seen in the absence of evidence of heart disease. It appears to us that the assumption that it is a normal variant is preferable, since it would tend to agree with electrocardiographic studies in which normal electrocardiograms are more often seen with cardiac disease than are abnormal electrocardiograms in the absence of heart disease.[7] Nevertheless, in individual cases the occurrence even of the otherwise normal S-type record should suggest thorough investigation of the case before dismissing it as a normal variant.

SUMMARY AND CONCLUSIONS

1. Electrocardiograms exhibiting prominent S waves (final inverted phase of the QRS complex measuring 25 per cent or more of the upright phase) in Leads I, II, and III were present in 84 cases of 1,850 consecutive electrocardiograms reviewed.

2. In 41 of these cases definite electrocardiographic abnormalities, such as left ventricular preponderance, right ventricular preponderance, combined right and left ventricular strain, myocardial infarction, and nonspecific abnormal patterns were found. In 8 others questionable abnormalities were present.

3. In 35 cases no other deviations from the normal pattern were observed and these were regarded as the otherwise normal S type of electrocardiogram. The criteria employed in this deduction are described. In 19 of these cases no demonstrable heart disease was present; in 3 the clinical findings were inconclusive; and in 13 there was clinical evidence of heart disease.

4. It is concluded that electrocardiograms exhibiting prominent S waves in Leads I, II, and III are more common in patients with evidence of heart disease than in normal persons in the population of an electrocardiographic laboratory. However, in an otherwise normal electrocardiogram the S type may be a normal variant, but before this decision is made the case should be thoroughly investigated.

We are grateful to Dr. L. N. Katz for his invaluable advice and criticism in this study.

REFERENCES

1. Ashman, R., and Hidden, E. H.: Rightward Deviation of the Axis of the T-Wave as an Index of Myocardial Disease, Ann. Int. Med. 12: 1682, 1939.
2. Ashman, R., and Hull, E.: Essentials of Electrocardiography, ed. 2, New York, 1941, The Macmillan Co., p. 128.
3. Katz, L. N., Goldman, A. M., Langendorf, R., Kaplan, L. G., and Killian, S. T.: The Diagnostic Value of the Electrocardiogram Based on an Analysis of 149 Autopsied Cases, Am. Heart J. 24: 627, 1942.
4. Hurwitz, M. M., Langendorf, R., and Katz, L. N.: The Diagnostic QRS Patterns in Myocardial Infarction. Unpublished.
5. Langendorf, R. L., Hurwitz, M. M., and Katz, L. N.: The Electrocardiographic Patterns of Combined Ventricular Strain, Brit. Heart J. In Press.
6. Katz, L. N.: Electrocardiography, Philadelphia, 1941, Lea & Febiger.
7. Pardee, H. E. B., and Price, L.: Relation of Myocardial Disease to Abnormalities of the Ventricular Complex of the Electrocardiogram, Am. Heart J. 15: 28, 1938.

ELECTROLYTE AND WATER EXCHANGE BETWEEN SKELETAL MUSCLE, "AVAILABLE (THIOCYANATE) FLUID," AND PLASMA IN THE DOG FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE*

David M. Harkness, B.S., Edward Muntwyler, Ph.D., and Frederick R. Mautz, M.D., Cleveland, Ohio, and Robert C. Mellors, Ph.D., Baltimore, Md.

SHORTLY following the introduction of desoxycorticosterone acetate for clinical use in 1939, numerous studies appeared dealing with its therapeutic value in the treatment of Addison's disease and with its effect on electrolyte and water metabolism. These studies[1-6] revealed, among other things, that the administration of the substance to patients with Addison's disease or to animals in adrenal insufficiency leads to a retention of sodium and chloride, and to an increased excretion of potassium and phosphorus. The plasma sodium and chloride concentrations become elevated to normal, and the plasma and extracellular fluid volumes become increased. At the same time the plasma potassium becomes reduced, frequently to abnormally low levels. While many clinical studies showed favorable therapeutic results with the use of desoxycorticosterone acetate, a number of authors [1, 3, 7, 8] issued a warning that symptoms of overdosage may occur in certain patients, particularly those receiving high sodium and low potassium diets. Indications of an excessive dosage of the substance or of sodium chloride, or of both, may be found in the appearance of edema, rapid gain in weight, increase in blood pressure, and finally in signs of congestive heart failure. The administration of large doses of desoxycorticosterone acetate to normal dogs has not been found to lead to the above symptoms; however, such animals develop a syndrome resembling that of diabetes insipidus[9-11] and show periodic attacks of muscular weakness or paralysis.[10, 12]

The experiments included in this paper were undertaken for the following purposes: (1) to observe any changes of the plasma volume and "available (thiocyanate) fluid" from normal; and (2) to observe the electrolyte and water exchanges between skeletal muscle and plasma following the administration of the drug in large doses and after its discontinuance.

METHODS AND CALCULATION

Adult male dogs were used in this study and were placed on a constant diet (diet suggested by Nilson[13] and supplemented by 15 Gm. of sodium chloride

*From the Departments of Biochemistry and Surgery, School of Medicine, Western Reserve University, Cleveland.

Aided by a grant from the John and Mary R. Markle Foundation.

A preliminary report upon this work was presented before the meeting of the American Society of Biological Chemists at Boston, March, 1942 (Federation Proceedings 1: 114, 1942).

Received for publication, August 7, 1942.

and 5 Gm. of sodium citrate per day) for a period of at least seven days before the experiments were undertaken. It was customary to withhold food for eighteen to twenty-four hours before collecting the samples for analysis. Control determinations of the plasma volume and the "available (thiocyanate) fluid" were made by the direct method of Gregersen and Stewart,[14] as adapted to the photoelectric colorimeter by Gibson and Evelyn.[15] The animals then received desoxycorticosterone acetate* subcutaneously (1 mg. per kilogram of body weight daily) for a period of fourteen days, at which time the above determinations were repeated. Immediately following these determinations, blood was withdrawn under oil from the femoral artery, placed under oil in a special centrifuge tube containing heparin to prevent clotting, and centrifuged at once for the plasma analyses. Skeletal muscle (lumbar portion, sacrospinalis) was obtained under sodium pentobarbital anesthesia as quickly as possible following the collection of the blood and was sampled for the several analyses in the manner previously described.[16] The following determinations were carried out on the plasma: water, chloride, sodium and potassium; on the muscle: water, chloride, sodium, potassium, and total neutral fat. The chemical methods were the same as those employed in previous studies.[17]

The administration of the desoxycorticosterone acetate was then discontinued, and after a period of fourteen days the foregoing observations were repeated. During this interval the diet remained unchanged in three of the animals while in five others the salt supplement was changed to 3 Gm. of sodium chloride plus 10 Gm. of potassium dihydrogen phosphate daily.

The volumes of the extracellular (F) and intracellular (C) phases of muscle were calculated in the manner outlined by Hastings and Eichelberger,[18] which is based upon the assumption that all the chloride is extracellular and is present at a concentration equal to that of an ultrafiltrate of plasma. In order to approximate the volume changes produced in the extracellular and intracellular phases of 1 kilogram of original muscle, the calculations were made relative to a control series, assuming a constant solid content of the intracellular phase. The partition of sodium and potassium between the extracellular and intracellular phases of muscle was carried out by the method described by previous authors.[18, 19] The experimental values herein reported for intracellular sodium and potassium are given in terms of a kilogram of intracellular water and have been corrected to an intracellular solid content equal to that of the control mean.

RESULTS

Changes of the Plasma Volume and "Available (Thiocyanate) Fluid."— Measurements of plasma volume and "available (thiocyanate) fluid" were carried out on nine animals following the administration of desoxycorticosterone acetate and on eight animals after its discontinuance. On the basis of individual control levels, the plasma volume was found to be increased by an average of 11 per cent and to range from 1 to 33 per cent, with an increase greater than 10 per cent occurring in four instances. (The increase of plasma volume amounted, on the average, to 77 c.c., with a range of variation from 8 to 206 c.c.). Likewise the "available (thiocyanate) fluid" was found to be increased

*We are indebted to the Schering Corporation for the generous supply of crystalline desoxycorticosterone acetate in sesame oil (Cortate) which was employed for these studies.

by an average of 15 per cent and to range from 5 to 40 per cent, with an increase greater than 10 per cent occurring in five instances. (The increase of "available (thiocyanate) fluid" amounted, on the average, to 700 c.c., with a range of variation from 290 to 1,600 c.c.)

Fourteen days after the administration of the drug had been discontinued, there was found to be a persistence of the average increase of the plasma volume and, although less, of the "available (thiocyanate) fluid." Thus the plasma volume was found to be increased by an average of 16 per cent and to range from 2 to 42 per cent, with an increase greater than 10 per cent occurring in five instances. Similarly, the "available (thiocyanate) fluid" was found to be increased, on the average, by 7 per cent and to range from –5 to 21 per cent, with values greater than 10 per cent occurring in only three instances.

From these data it would appear that, although in a fairly high percentage of animals receiving large doses of desoxycorticosterone acetate the increases of plasma volume and "available (thiocyanate) fluid" may not exceed the possible experimental error, there does seem to be a definite tendency for both volumes to become elevated and in some instances to become elevated appreciably.

TABLE I

ELECTROLYTE AND WATER CONTENT OF PLASMA FROM DOGS FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE AND AFTER ITS DISCONTINUANCE*

	WATER	CHLORIDE	SODIUM	POTASSIUM
	Gm. per kg.	meq. per kg. water		
Controls (17)				
Mean	918.4	120.5	156.3	3.1
Standard deviation	7.1	2.9	3.5	0.6
Following desoxycorticosterone acetate (10)				
Mean	920.2	114.6	157.4	2.1
Maximum	926	118	161	3.1
Minimum	915	111	152	1.6
After desoxycorticosterone acetate discontinuance (3)†				
Mean	914.5	117.1	159.2	2.2
Maximum	920	118	163	2.7
Minimum	908	116	156	2.0
After desoxycorticosterone acetate discontinuance (5)‡				
Mean	918.3	116.4	156.3	2.6
Maximum	923	119	162	3.4
Minimum	913	115	153	2.2

*Two-week periods. One milligram per kilogram of body weight of desoxycorticosterone acetate was administered daily. The numbers within the parentheses indicate the number of animals.

†High sodium and low potassium diet.

‡Low sodium and moderately high potassium diet.

Plasma Changes.—The results of the analysis of plasma are presented in Table I. By comparison with a control series[2] it will be seen that the outstanding changes encountered following the administration of desoxycorticosterone acetate were the deficits in the average levels of potassium and chloride. That there was a tendency to an elevated plasma sodium concentration was indicated by the slightly elevated average value.

Two weeks after the administration of the drug had been discontinued, the plasma sodium showed, on the average, a further increase in the three animals whose diet was left unchanged, while it fell back to the control mean in the

five animals whose salt supplement was changed to 3 Gm. of sodium chloride plus 10 Gm. of potassium dihydrogen phosphate daily. Some decrease of the average levels for both potassium and chloride was still found to be present in both groups of animals.

Attention should be called to the fact that Kuhlman, Ragan, and associates,[12] and Ferrebee, Parker, and others[10] noted an increase of the plasma sodium concentration amounting to about 4 or 5 meq. per liter and a fall of the plasma potassium of about 1 to 1.5 meq. per liter in normal dogs receiving 25 mg. of desoxycorticosterone acetate daily for periods of twenty or more days. Although the changes of the plasma sodium were less striking, the deficits of plasma potassium encountered in the present experiments are of the order noted by the above workers. It is of some interest that even though the plasma potassium tended to return to normal, the average figure was still lowered somewhat two weeks after the discontinuance of the drug despite the added daily intake of potassium.

TABLE II

ELECTROLYTE AND WATER CONTENT OF SKELETAL MUSCLE FROM DOGS FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE*

(The values are expressed in terms of 1,000 Gm. of fat-free muscle.)

	CONTROLS (17)		FOLLOWING DESOXYCORTICOSTERONE ACETATE ADMINISTRATION (10)		
	MEAN	STANDARD DEVIATION	MEAN	MAXIMUM	MINIMUM
Chloride, meq.	19.8	2.0	20.0	22	19
Sodium, meq.	31.1	3.1	46.1	57	39
Potassium, meq.	88.5	6.0	66.5	76	52
Water, Gm.	764.0	8.4	759.8	776	749
Extracellular phase, (F), Gm.	158	15	168	180	146
Intracellular water, $[H_2O]_c$,† Gm.	722.3	9.7	713.8	732	702
Intracellular sodium, $[\tilde{Na}]_c$,‡ meq.	12.7	4.7	35.6	54	21
Intracellular potassium, $[K]_c$,‡ meq.	145.0	11.7	111.2	124	88
ΔM, Gm.			-16	-60	55
ΔC, Gm.			-23	-59	30
ΔF, Gm.			7	30	-19

*Lumbar portion, sacrospinalis. One milligram per kilogram of body weight of desoxycorticosterone acetate was injected daily for fourteen days.

†Per 1,000 Gm. of muscle cells.

‡Per 1,000 Gm. of intracellular water, corrected to a solid content equal to that of the control. Numbers within the parentheses indicate the number of animals.

Skeletal Muscle Changes.—The results of the analysis of skeletal muscle have been presented in Tables II and III. By comparing these data with those of a control series,* it will be observed that two weeks following the administration of desoxycorticosterone acetate (Table II) there was a striking fall of skeletal muscle potassium and a gain of sodium. The muscle chloride remained unchanged while the average water content was lowered slightly.

The partition of sodium and potassium between the extracellular and intracellular compartments of skeletal muscle revealed that the "excess" sodium, which can be located in the intracellular phase, $[Na]_c$, increased from a control level of 12.7 meq., ±4.7 meq., per kilogram of intracellular water to an average figure of 35.6 meq., with a range of variation from 21 to 54 meq. Simultaneously, the average intracellular potassium per kilogram intracellular water, $[K]_c$,

*The data for plasma and skeletal muscle of the control series were reported in connection with another study.[11] These observations were carried out on a separate series of animals; however, the chemical methods and management of the animals were identical with those employed here.

fell from the control level of 145.0 meq., ±11.7 meq., to 111.2 meq., with a range of variation from 88 to 124 meq. It will be observed that, on the average, the sum of the concentrations $[Na]_c$ and $[K]_c$ become lowered by 10.9 meq., indicating that the gain of "excess" sodium did not completely compensate the loss of intracellular potassium. In this connection it will be seen that the calculations of the volume changes produced in the extracellular and intracellular phases of 1 kilogram of control muscle revealed some irregularity. However, on the basis of an average 23 Gm. decrease in the intracellular phase, ΔC, and an average amounted to a decrease of 16 Gm. per kilogram of control muscle, ΔM, consisting of an average 23 Gm. decrease in the intracellular phase, ΔC, and an average 7 Gm. increase in the extracellular phase, ΔF. Thus, despite a marked divergence from normal of the electrolyte pattern of the intracellular phase, there were, on the average, no striking changes from normal in the volume relationship between the extracellular and intracellular phases. In fact, the average volume changes encountered were in the direction of those which might be anticipated on the basis of a gain of the extracellular sodium concentration and of the "available (thiocyanate) fluid."

TABLE III

ELECTROLYTE AND WATER CONTENT OF SKELETAL MUSCLE FROM DOGS AFTER THE DISCONTINUANCE OF DESOXYCORTICOSTERONE ACETATE ADMINISTRATION*

(The values are expressed in terms of 1,000 Gm. of fat-free muscle.)

	HIGH SODIUM—LOW POTASSIUM DIET (3)			LOW SODIUM—MODERATE POTASSIUM DIET (5)		
	MEAN	MAXIMUM	MINIMUM	MEAN	MAXIMUM	MINIMUM
Chloride, meq.	20.7	23	18	20.0	24	16
Sodium, meq.	46.9	53	39	40.1	45	30
Potassium, meq.	61.4	63	60	72.5	83	64
Water, Gm.	762.0	775	748	765.5	773	759
Extracellular phase, (F), Gm.	170	183	147	165	193	135
Intracellular water, $[H_2O]_c$,† Gm.	715.8	728	707	721.2	732	711
Intracellular sodium, $[Na]_c$,‡ meq.	35.9	42	29	25.9	36	15
Intracellular potassium, $[K]_c$,‡ meq.	102.7	104	99	119.6	132	105
ΔM, Gm.	-6	-66	49	7	41	-22
ΔC, Gm.	-17	-45	19	-1	-35	34
ΔF, Gm.	11	30	-21	8	37	-21

*Lumbar portion, sacrospinalis. Observations were made fourteen days after discontinuance of desoxycorticosterone acetate administration.

†Per 1,000 Gm. of muscle cells.

‡Per 1,000 Gm. of intracellular water, corrected to a solid content equal to that of the control. Numbers within the parentheses indicate the number of animals.

By comparing the data presented in Table III with those given in Table II, it will be observed that a marked deviation from normal of the skeletal muscle sodium and potassium content was found to persist in the animals studied two weeks after the administration of the drug had been discontinued. The partition of sodium and potassium between the extracellular and intracellular compartments revealed, on the average, no tendency of the intracellular concentrations of these two cations to return to normal levels in the three animals which were permitted to continue on the diet high in sodium and low in potassium. However, in the case of the five animals which received a daily salt supplement of 3 Gm. of sodium chloride plus 10 Gm. of potassium dihydrogen phosphate during the period of drug discontinuance, a tendency for the intracellular concentrations of sodium and potassium to return to normal was noted. Thus in the latter animals

the average intracellular sodium, $[Na]_c$, was 9.7 meq. lower, and the average intracellular potassium, $[K]_c$, was 8.4 meq. higher than the average levels observed after the administration of the drug. It will be observed that as was the case with the muscles of the animals examined after the administration of the desoxycorticosterone acetate, the calculations of the volume changes produced in the extracellular and intracellular phases of 1 kilogram of control muscle revealed, on the average, no changes from normal which could not be anticipated on the basis of the extracellular sodium concentration and the "available (thiocyanate) fluid."

DISCUSSION

At the time the present experiments were being brought to a conclusion, the report by Ferrebee, Parker, Carnes, Gerity, Atchley, and Loeb[10] appeared, calling attention to the fact that normal dogs receiving daily injections of 25 mg. of desoxycorticosterone acetate develop muscular weakness and a syndrome resembling diabetes insipidus. It was pointed out, further, that there were attacks of paralysis associated with a partial replacement of the intracellular potassium of the skeletal muscles by sodium. In respect to the distortion from normal, the changes of intracellular sodium and potassium of skeletal muscle encountered in the present study are in complete harmony with those reported by the aforementioned workers. Even though smaller doses were employed in the present experiments, two of the ten dogs exhibited paralysis while receiving the drug. Interestingly enough, these two animals also showed the greatest losses of intracellular potassium and gains of intracellular sodium, the figures for $[K]_c$ being 87.7 and 96.0 meq., and those for $[Na]_c$ being 54.4 and 47.4 meq. per kilogram of intracellular water, respectively.

SUMMARY

Changes of the plasma volume, the "available (thiocyanate) fluid," and the electrolyte and water content of plasma and skeletal muscle were observed in normal dogs following the daily injection (1 mg. per kilogram of body weight) of desoxycorticosterone acetate for a period of two weeks and two weeks after the administration of the drug had been discontinued. The pertinent findings may be summarized as follows:

1. Normal dogs maintained on a high sodium-low potassium diet exhibited a tendency for both the plasma volume and the "available (thiocyanate) fluid" to become increased while receiving the drug for a period of two weeks. In many instances the increases of plasma volume and "available (thiocyanate) fluid" did not exceed the possible experimental error. However, not infrequently both volumes were found to be elevated appreciably.

2. Two weeks after the administration of the drug the animals showed, on the average, a fall of the plasma potassium and chloride concentrations and a tendency toward an elevated plasma sodium concentration. At the same time there was a striking reduction of the skeletal muscle potassium content, a gain of sodium, and no change of the skeletal muscle chloride. On the average, the loss of intracellular potassium was not completely compensated by a gain of the sodium which could be located in the intracellular phase.

3. The animals which were studied two weeks after the administration of the drug had been discontinued continued to show a marked reduction of the skeletal

muscle potassium and elevation of sodium. While there was no tendency for the intracellular concentrations of these two cations to return to normal levels in animals maintained on a high sodium-low potassium diet during this period, there was, on the average, such a tendency in the animals receiving a lowered sodium and increased potassium intake.

4. Despite the marked divergence of the electrolyte pattern of the intracellular phase from the normal, there were, on the average, no striking changes from normal in the volume relationship between the extracellular and intracellular phases.

REFERENCES

1. Ferrebee, J. W., Ragan, C., Atchley, D. W., and Loeb, R. F.: Desoxycorticosterone Esters. Certain Effects in the Treatment of Addison's Disease, J. A. M. A. **113**: 1725, 1939.
2. Loeb, R. F.: Adrenal Cortex Insufficiency, J. A. M. A. **116**: 2495, 1941.
3. Thorn, G. W., Howard, R. P., and Emerson, K., Jr.: Treatment of Addison's Disease With Desoxy-Corticosterone Acetate, A Synthetic Adrenal Cortical Hormone (Preliminary Report), J. Clin. Investigation **18**: 449, 1939.
4. Thorn, G. W., Engel, L. L., and Eisenberg, H.: Treatment of Adrenal Insufficiency by Means of Subcutaneous Implants of Pellets of Desoxycorticosterone Acetate (A Synthetic Adrenal Cortical Hormone), Bull. Johns Hopkins Hosp. **64**: 155, 1939.
5. Thorn, G. W., Howard, R. P., Emerson, K., Jr., and Firor, W. M.: Treatment of Addison's Disease With Pellets of Crystalline Adrenal Cortical Hormone (Synthetic Desoxycorticosterone Acetate) Implanted Subcutaneously, Bull. Johns Hopkins Hosp. **64**: 339, 1939.
6. Wells, B. B., and Kendall, E. C.: A Quantitative Difference in the Effect of Compounds Separated From the Adrenal Cortex on Distribution of Electrolytes and on Atrophy of the Adrenal and Thymus of Rats, Proc. Staff Meet., Mayo Clin. **15**: 133, 1940.
7. McGavack, T. H.: Adrenal Insufficiency: Some Pitfalls in the Treatment of Addison's Disease, J. Clin. Endocrinol. **1**: 68, 1941.
8. Willson, D. M., Rynearson, E. H., and Dry, T. J.: Cardiac Failure Following Treatment of Addison's Disease With Desoxycorticosterone Acetate, Proc. Staff Meet., Mayo Clin. **16**: 168, 1941.
9. Ragan, C., Ferrebee, J. W., Phyfe, P., Atchley, D. W., and Loeb, R. F.: A Syndrome of Polydipsia and Polyuria Induced in Normal Animals by Desoxycorticosterone Acetate, Am. J. Physiol. **131**: 73, 1940.
10. Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F.: Certain Effects of Desoxycorticosterone. The Development of "Diabetes Insipidus" and the Replacement of Muscle Potassium by Sodium in Normal Dogs, Am. J. Physiol. **135**: 230, 1941.
11. Mulinos, M. G., Spingarn, C. L., and Lojkin, M. E.: A Diabetes Insipiduslike condition Produced by Small Doses of Desoxycorticosterone Acetate in Dogs, Am. J. Physiol. **135**: 102, 1941.
12. Kuhlman, D., Ragan, C., Ferrebee, J. W., Atchley, D. W., and Loeb, R. F.: Toxic Effects of Desoxycorticosterone Esters in Dogs, Science **90**: 496, 1939.
13. Nilson, H. W.: Corticoadrenal Insufficiency: Metabolism Studies on Potassium, Sodium and Chloride, Am. J. Physiol. **118**: 620, 1937.
14. Gregersen, M. I., and Stewart, J. D.: Simultaneous Determination of the Plasma Volume With T-1824 and the "Available Fluid" Volume With Sodium Thiocyanate, Am. J. Physiol. **125**: 142, 1939.
15. Gibson, J. G., 2d, and Evelyn, K. A.: Clinical Studies of the Blood Volume, IV. Adaptations of the Method to the Photoelectric Microcolorimeter, J. Clin. Investigation **17**: 153, 1938.
16. Muntwyler, E., Mellors, R. C., Mautz, F. R., and Mangun, G. H.: Electrolyte and Water Equilibria in the Dog. II. Electrolyte and Water Exchange Between Skeletal Muscle and Blood in Adrenal Insufficiency, J. Biol. Chem. **134**: 367, 1940.
17. Mellors, R. C., Muntwyler, E., and Mautz, F. R.: Electrolyte and Water Exchange Between Skeletal Muscle and Plasma in the Dog Following Acute and Prolonged Extracellular Electrolyte Loss, J. Biol. Chem. **144**: 773, 1942.
18. Hastings, A. B., and Eichelberger, L.: The Exchange of Salt and Water Between Muscle and Blood. I. The Effect of an Increase in Total Body Water Produced by the Intravenous Injection of Isotonic Salt Solutions, J. Biol. Chem. **17**: 73, 1937.
19. Harrison, H. E., Darrow, D. C., and Yannet, H.: The Total Electrolyte Content of Animals and Its Probable Relation to the Distribution of Body Water, J. Biol. Chem. **113**: 515, 1936.

LABORATORY METHODS

GENERAL

USE OF SODIUM DESOXYCHOLATE-LYSED ANTIGEN FOR THE PRODUCTION OF PNEUMOCOCCUS ANTISERUM*

Herman A. Iverson, M.D., and Loring W. Pratt, A.B.
Baltimore, Md.

THE production of antibodies against pneumococci by the use of lysates of pneumococci has been demonstrated by a number of investigators. Meyer,[1] and Meyer and Sukneff,[2] used a sodium taurocholate lysate of type I pneumococci to immunize rabbits and mice. Pneumococci treated with soaps (sodium ricinoleate) were used by Larson and Nelson[3] and by Larson[4] for prophylaxis in man and to produce a therapeutic antiserum. Pneumococci treated with bile salts were used as antigens by some workers.[5] White,[6] summarizing previous work, says: "Filtrates from fluid cultures and watery or saline extracts of pneumococci, representing as they do only a part of the antigenic components of the pneumococcal cell, are more limited in immunizing properties than the entire cell," and further, "while pneumococci treated with bile salts may induce immunity in mice and rabbits, and presumably in other animals, the immunity is of low order and is lacking in type specificity."

Our experiments were made with antigens prepared in three ways: 1. *Heat-killed whole cultures* of young virulent strains of type I pneumococci were made into antigens according to the method of the Massachusetts Antitoxin and Vaccine Laboratory (quoted by White). 2. *Lysate antigens,* made from sixteen-hour broth cultures of the same strains, washed with salt solution, and diluted with salt solution until the turbidity of the suspension corresponded to tube 3 of a barium sulfate standard.[8] The suspension was then lysed by adding 2 drops of sodium desoxycholate solution[9] to each 8.0 c.c. of the culture suspension. 3. *Lysate antigens,* prepared as described and *passed through a Seitz filter* to remove any possibility of production of antibodies being the result of particles of the cells that failed to dissolve. All antigens were prepared fresh daily.

Some of the rabbits were given intravenous injections of antigens for twelve consecutive days; an initial injection of 0.5 c.c., increased each day by 0.5 c.c., until a maximum daily dose of 2.0 c.c. was attained. One group of rabbits was injected according to the protocol of the Massachusetts Antitoxin and Vaccine Laboratory, consisting of a total of 18 injections given over a period of twelve weeks. Other rabbits were given a total of 12 injections in series of four con-

*From the Department of Pathology and Bacteriology, the Johns Hopkins University School of Medicine, Baltimore.
This work was supported in part by a grant from Hynson, Westcott & Dunning, Baltimore.
Received for publication, May 5, 1942.

secutive days with seven-day rest periods between series. In the above experiments no filtered-lysed antigen was used. In still another group of rabbits, however, two animals were injected with heat-killed antigen, two with lysed antigen, and two with filtered-lysed antigen. Four daily injections were given, followed by a rest period of thirty-one days and four more daily injections. All animals were bled five days after the last injection. No significant difference was obtained when the same type of antigen was used in the above methods of immunization. It would appear, therefore, that the shorter period of immunization is to be preferred.

RESULTS

The potency of the antisera was determined by means of agglutination tests, Neufeld tests, microscopic precipitin tests by the method of Schaub and Reid,[7] and mouse protection tests according to the potency (of serum) test of the National Institute of Health. The results can be summarized in Table I. They were similar in all trials except for individual variations in the rabbits.

TABLE I

TEST	HEAT-KILLED ANTIGEN	LYSED PNEUMOCOCCUS ANTIGEN	LYSED AND FILTERED ANTIGEN
Agglutination tests	Complete in 1:320 dilution	Complete in 1:320 dilution	Not done
Neufeld tests	Slight "Quellung"	Slight "Quellung"	Slight "Quellung"
Microscopic precipitin tests	Unsatisfactory	Unsatisfactory	Unsatisfactory
Mouse protection tests	Protection against as much as 10 million L.D.	Protection against as much as 1 million L.D.	Protection against as much as 1 million L.D.

DISCUSSION

The agglutination and mouse protection titers of sera from rabbits injected twelve times on consecutive days with lysate antigen compared favorably with that of sera from rabbits given a heat-killed antigen either as 18 injections over twelve weeks or 12 injections on as many days. Four lysate immunized animals gave lower titers than any of those immunized with heat-killed antigen. In a couple of instances lower titers were obtained in animals immunized with heat-killed antigen than were obtained in those giving best results with lysed antigen. Animals immunized with filtrate from lysed cultures produced as high titer serum as those injected with lysate not filtered.

The specificity of antiserum produced in rabbits by injecting sodium desoxycholate-lysed antigen is nearly equal, in these experiments, to that of antiserum produced in rabbits with heat-killed antigens.

No apparent systemic harm was done to the rabbits by the sodium desoxycholate. Local necrosis and edema occurred when the sodium desoxycholate antigens entered directly into the tissues of the rabbit.

CONCLUSIONS

1. Sera from rabbits immunized with lysate, filtered or not, freshly prepared by treating young virulent cultures of type I pneumococci with sodium desoxy-

cholate, gave specific mouse protection and showed some ability to cause agglutination and "Quellung" in specific type sera but gave no group reaction.

2. The sodium desoxycholate-lysed pneumococci produced, in a short time, in rabbits, antisera of high titer and possessing type specificity.

REFERENCES

1. Meyer, H.: Über aktive Immunisierung von Mäusen durch in Natrium taurocholicum gelöste Pneumokokken, Ztschr. f. Hyg. u. Infektionskr. 107: 416, 1927.
2. Meyer, H., and Sukneff, W. W.: Über die immunisierende Wirkung von avirulenten Pneumokokken und von Kulturfiltraten und Auflosungen virulenter Pneumokokken, Ztschr. f. Hyg. u. Infektionskr. 109: 78, 1928.
3. Larson, W. P., and Nelson, E.: The Antigenic Properties of Pneumococci and Streptococci Treated With Sodium Ricinoleate, Proc. Soc. Exper. Biol. & Med. 22: 357, 1925.
4. Larson, W. P.: Cross Protection of Animals Immunized With Various Pneumococcus Types. (Read before the Am. A. Path. & Bact., 1934. Quoted from White.[6])
5. Ziegler, E. E.: Some Properties of Pneumocholin, A Biochemical Antigen, J. Lab. & Clin. Med. 18: 695, 1933.
6. White, Benjamin: The Biology of the Pneumococcus, New York, 1938, The Commonwealth Fund.
7. Schaub, I. G., and Reid, R. D.: A Microscopic Precipitin Test for Typing Pneumococci, J. A. M. A. 111: 1285, 1938.
8. Zinsser, H., and Bayne-Jones, S.: A Textbook of Bacteriology, ed. 8, New York, 1939, D. Appleton-Century Co., page 938.
9. Leifson, E.: The Use of Sodium Desoxycholate for the Identification of Pneumococci, J. A. M. A. 104: 213, 1935.

THE CYTOLOGY OF THE POLYMORPHONUCLEAR LEUCOCYTE IN TOXIC CONDITIONS*

Eric Ponder, M.D., D.Sc., and Ruth van Olinda Ponder, B.A.
Mineola, L. I.

FROM time to time it has been suggested that valuable information can be obtained by considering the detailed cytology of the white blood cells in infective states, altogether apart from that given by the form of the polymorph nuclei, which is the basis of the Arneth count, the polynuclear count, and their modifications. More specifically, many observers from the time of Bodkin (1892) and Ewing (1901) have been impressed by the degenerative changes in the polymorphs seen in severe toxemias, and Strumia (1936) has classified the degenerated cells into those showing "toxic granules" (Cesaris-Demil, 1908), those with clumped granules and vacuolation, and those showing "coagulation necrosis," edema, and nuclear rupture. Taking these cytological appearances along with the "nonfilamented count" (a simplified polynuclear count, the nonfilamented cells being equivalent to cells of Class I) Amidon (1939) has tried to relate the changes in white blood cell cytology to the clinical course in toxic states (appendicitis, pneumonia, etc.), but is not enthusiastic about the practicability of the procedure, nor certain of the significance of the results.

No one who examines large numbers of properly stained blood films for the purpose of making routine differential or polynuclear counts can fail to be impressed by the variations in white blood cell cytology which present themselves,

*From the Nassau Hospital, Mineola.
Received for publication, May 19, 1942.

but the difficulty is to express what one sees in such a way as to give the various cytological appearances (toxic granulation, vacuolation, etc.) their proper weight, so that the final result is in some way related to the clinical state of the patient. As an example of the difficulty, one soon finds that it is quite unsatisfactory to give the percentage number of cells with toxic granulations as opposed to the number without them, for the division is not sufficiently distinct, and the cells with toxic granules vary greatly with respect to the degree of granulation. Similarly, an enumeration of degenerated, as opposed to undegenerated, cells does not allow for the fact that there are many degrees of degeneration. Still further difficulty results when one considers two attributes, such as toxic granulation and degeneracy, together, for a degenerate cell may or may not have toxic granules; such attempts at an all-or-none classification only lead to Amidon's conclusion that attention to these cytological details is more trouble than it is worth.

These difficulties largely disappear if one gives up trying to enumerate all-or-none characteristics (percentage of vacuolated cells, percentage of cells with toxic granules, etc.), and substitutes an estimate of the extent to which each abnormal attribute (vacuolation, toxic granulation, etc.) appears in the average cell, describing the extent by plus signs or numbers, and forming an impression of the "average cell" while examining the film for other purposes, e.g., while making a differential count or polynuclear count.* This report is concerned with the results of applying this method to several thousand blood films over about a year's time, during which the method was being tested for its practicability.

STAINING METHODS

1. *Preparation of glassware.* New, or at least unetched, slides are washed in a strong solution of Gold Dust for hours or even days, and are rinsed thoroughly and individually in hot running water. They are then dipped in 95 per cent alcohol, wiped dry with a clean towel, and stored in covered boxes. Cover slips are cleaned in the same way. One should not use cleaning solution or acid alcohol, because anomalies in the staining reaction follow.

2. *Staining.* Films are made in the usual way from fresh blood, and with properly cleaned slides it is easy to prepare thin and even films. Heparinized blood can be used if fresh, but oxalated blood should not be used. The film is allowed to dry in air, and is fixed for five minutes in methyl alcohol in a Coplin jar. The methyl alcohol must be "absolute, acetone free," and should be frequently replaced; if this is not done, anomalies in staining result. The fixed film is dried again in air before the application of the stain. Giemsa blood stain, stock solution, is diluted 1:10 with distilled water, the dilution being made just

*Osgood and Ashworth illustrate toxic neutrophiles in their *Atlas of Hematology*, Nos. 87 to 92. They describe three cytological changes in toxic conditions: basophilia of cytoplasm, the presence of vacuoles, and the presence of toxic granules. These they grade from one plus to four plus, and they say that death occurred in 100 consecutive cases in which two of these factors were graded from three to four plus. On the system of grading described in this paper, three plus and four plus are of less serious prognostic significance than on theirs. It will be noticed that their system does not include a grading of the feature "pyknotic nuclei," because this feature is not so clearly observed with Wright's stain, which Osgood and Ashworth use. They continue: "Evaluation of toxic changes is more reliable in prognosis than either the staff cell count or the ratio of neutrophiles to total polymorphs, but the use of all these criteria is worth while. Rosenthal and Sutra count the percentage of polymorphs showing toxic granules and report the result as the 'degenerative index.' We have had better results by evaluating all three toxic changes, and it is simpler to report these on a scale of one plus to four plus as one is doing the differential count." This corresponds to our experience.

before the stain is to be used; buffering is unnecessary if the water is freshly distilled. The slide, placed film-side up on a staining rack, is flooded with the diluted stain. The duration of staining is most important, and should be neither more nor less than thirty minutes, at the end of which time the film is rinsed with running distilled water for fifteen seconds, flooded with distilled water for thirty seconds, and finally tipped up on end to dry in air. The back of the slide is polished while it is still wet, and the examination of the cells is carried out with an oil-immersion objective in the usual way on the unmounted film. The slightest variation from this standard procedure will result in changes in the staining reaction, and, as a control on the staining method, one may use a film of one's own blood.

GRADING OF CYTOLOGICAL FEATURES

One hundred polymorphs are examined, as in the making of a polynuclear count, which, indeed, is done on every film, as described by Cooke and Ponder (1928). As each polymorph is seen, it is carefully examined and mentally classified as regards four features other than nuclear configuration: (1) whether the cytoplasmic granules are large, separated, and deeply staining, metachromatic or azurophile ("toxic granulation"); (2) whether the cell outline is sharp and circular, or irregular and presenting pseudopods ("ameboid"); (3) whether the cytoplasm of the cell is vacuolated ("vacuolation"); and (4) whether the nucleus shows pyknotic areas ("pyknotic nuclei"). After such a survey the observer will be able to form an opinion of the cytology of the average cell with respect to four features: toxic granulation, ameboid, vacuolation, and pyknotic nuclei. He will be able to give a somewhat arbitrary grade to each of these features, from one plus to four plus, and it is a matter of experience that he can do so with a surprising degree of certainty.

All sorts of elaborate schemes could be devised for expressing the results of the survey, but we have found it sufficient to denote the occurrence of each abnormal feature by a plus sign (from one plus to four plus), and to add the number of plus signs together, averaging the number of plus signs for the features "ameboid" and "vacuolated." The result of the survey is then represented by a figure which varies from 0 to 12, the value 0 meaning that all the abnormal cytological features are absent, and the value 12 meaning that they are each present to a maximal extent. For example, if a film shows:

Toxic granulation	+++
Ameboid	+
Vacuolation	+
Pyknotic nuclei	++

it is described as a film of grade 6. Again, if a film shows the following features:

Toxic granulations	++++
Ameboid	++
Vacuolation	++++
Pyknotic nuclei	++

it is described as a film of grade 9. A normal film is obviously one of grade 0. This method of grading the films may seem rough and arbitrary, but, nevertheless, it expresses the results of the cytological survey in a remarkably satisfactory way.

SIGNIFICANCE OF THE CYTOLOGICAL CHANGES

Considerable experience in grading polymorphs has brought out a number of points which have a bearing on the significance of the cytological changes.

1. The changes are not of an all-or-none nature, some cells showing them and some not. They affect all the cells, although some more than others, and this results in the information given by the grade being very different from that given by the polynuclear count. The figures for the various classes of the polynuclear count give the proportion of young cells (Class I) to older cells (Classes II, III, IV, and V, in order of increasing age), and a left-handed deflection shows that there has been either an increased production of young cells as a result of marrow stimulation, or an increased destruction of the older cells, or a combination of the two. This corresponds, by analogy with what occurs in a population, to an increased birth rate, an increased death rate in the older classes, or to a combination of both, and is a change which is characteristic of the population as a whole rather than of any one member in it. The grade, on the other hand, is an expression of the average state of the cell, not as regards age, but as regards cytological appearance and the processes which determine it at the moment; to extend the analogy, this would roughly correspond to the average nutrition of the individuals of the population. Whereas a change in the polynuclear count expresses a disturbance of the production-destruction steady state, a change in the grade seems to be evidence of an alteration in the *milieu* in which the cells circulate, and this produces substantially the same changes in all the cells simultaneously. An excellent example of this can be seen in cases where films are made within a few hours of an accident resulting in extensive tissue damage; e.g., compound fracture of femora, two hours after accident: 29,700 leucocytes per cubic millimeter, 81 per cent polymorphs, polynuclear mean, 1.41, grade 6.* The cells of all the polynuclear classes, from I to V, and even polycytes if they are present, show the changes in cytology, although the large number of cells of Class I are young cells just liberated from the marrow, while the cells of the higher classes must have been normal inhabitants of the blood stream at the time of the accident. Perhaps the best of all instances of the rapid development of pyknotic nuclei in the polymorphs of all the polynuclear classes is to be found in the guinea pig after the injection of nucleic acid (Curphey and Ponder, 1941); the multilobulated polymorphs (as high as Class X) all develop pyknotic nuclei within an hour of the subcutaneous injection of 1 mg. of nucleic acid per kilogram, and regain their normal appearance from four to eight hours later.

2. Deflections of the polynuclear count without anything more than the most trifling change in the grade (e.g., from 0 to 1, or from 1 to 2) occur after inhalation anesthesia (ether, ethylene, etc.) and can be discounted.

3. While a left-handed deflection of the polynuclear count is an excellent measure of the severity of an acute infection, and while a subsequent right-handed shift is a reliable indication of recovery, it is well recognized that the count tends to become stabilized at a low figure (usually 1.2 to 1.5) in chronic infections or in toxic states of long duration. Under these circumstances the

*This case may be contrasted with one of hemorrhage from the radial artery, uncomplicated by toxic manifestations: 21,000 leucocytes per cubic millimeter, 90 per cent polymorphs, polynuclear mean 1.28, grade 0.

fixed polynuclear count does not reflect small changes in the severity of the toxemia, and the grade is a far more sensitive measure of variations in the toxic state. Changes in the grade from day to day are of great prognostic value, especially if there is a definite trend, and although these changes are ultimately followed by corresponding shifts in the polynuclear count, they constitute the better guide to progress and indication of the outcome (cf. footnote,* page 317).

4. The various cytological features do not always undergo alteration simultaneously. The appearance of vacuoles, however, is so constantly accompanied by an ameboid cell outline that the abnormal properties "vacuolation" and "ameboid" can be considered as two manifestations of the same underlying change, and each given only half the weight of an abnormal attribute in determining the grade. "Pyknotic nuclei" often occur without any other abnormal cytological feature being present, and almost invariably do so when the patient becomes either dehydrated or acidotic.

5. There are certain artifacts which may lead to misinterpretations if unrecognized. The most important of these are the artifacts introduced by variations in the staining method, and it cannot be too strongly emphasized that the system of grading described here is entirely dependent on the staining methods being carried out without modification. The most frequently occurring artifacts are the following: (a) On improperly cleaned slides, and particularly on slides cleaned with acid, cleaning solution, and acid alcohol, the cytoplasm appears pink and the nuclei show pyknotic areas. (b) Methyl alcohol which is not fresh produces the same artifacts as are seen on slides cleaned with acid solutions. (c) Films made from blood kept for some hours in the refrigerator are likely to show cells which are vacuolated and ameboid. (d) Films made from oxalated blood show shrunken white blood cells, the cytology of which is very different from that of cells from fresh or heparinized blood. (e) Cells in films from patients who have been treated with sulfonamide drugs, and particularly sulfanilamide, are ameboid and vacuolated.

CLINICAL ILLUSTRATIONS

Tables I, II, and III illustrate the results of using this method of grading.

Table I shows the total white blood cell count, the polymorphs percentage, the polynuclear count mean, and the grade in patients who showed no signs of toxemia clinically. I have used the preoperative figures for 12 consecutive cases in which partial gastrectomy or colectomy was performed, since these provide a good illustration of what is found in surgical cases without toxic manifestations. Inspection of Table I shows that the grade, rather than the total white cell count, the polymorph percentage, or the mean of the polynuclear count, is the reliable indication of the absence of toxemia.

Table II gives the same data in 12 cases in which there were clinical signs of severe toxemia. Again it will be apparent that the grade is a better indication of the toxic condition than the total white blood cell count, the polymorph percentage, or the polynuclear count.

Table III shows the values for the grade in a series of miscellaneous toxic conditions (corresponding roughly to Amidon's Table IV). By "first observation" is meant the observation on admission or at the time of the acute episode,

TABLE I

	TOTAL COUNT × 10^{-3}	POLYMORPHS %	POLYNUCLEAR MEAN*	GRADE
Carcinoma of cecum	11.2	69	2.21	0
Carcinoma of rectum	9.3	54	2.20	0
Stenosing gastric ulcer	4.7	42	2.16	1
Stenosing gastric ulcer	7.4	79	2.00	0
Stenosing gastric ulcer	9.2	58	1.98	0
Carcinoma of sigmoid	6.1	74	1.78	0
Stenosing gastric ulcer	8.4	64	1.75	1
Stenosing gastric ulcer	10.2	60	1.66	1
Carcinoma of sigmoid	7.7	65	1.66	1
Endometriosis of cecum	14.3	76	1.64	2
Carcinoma of hepatic flexure	5.9	64	1.39	3
Carcinoma of pylorus	14.1	82	1.36	2

*By the polynuclear mean is meant the average number of lobes per polymorph. It is computed in a count of 100 polymorphs by multiplying the number of cells with one lobe to the nucleus by 1, the number with two lobes by 2, the number with three lobes by 3, the number with four lobes by 4, and the number with five or more lobes by 5; the figures are added together and divided by 100. The resulting figure expresses in a general sort of way, what is expressed by writing out the figures for the count in extenso. The normal polynuclear mean ranges from 2.3 to 2.7; means less than this correspond to "left-handed deflections," and the most extreme deflection (100 cells of Class I) corresponds to a mean of 1.0

TABLE II

	TOTAL COUNT × 10^{-3}	POLYMORPHS %	POLYNUCLEAR MEAN	GRADE
Toxemia of pregnancy	7.6	66	2.49	10
Pelvic inflammatory disease	8.5	60	2.24	7
*Subacute endocarditis	3.9	81	1.77	9
*Subphrenic abscess	20.2	86	1.74	8
Diffuse peritonitis	17.4	93	1.71	9
Tuboovarian abscess	29.4	87	1.66	8
Acute pancreatitis	30.6	83	1.46	9
Rectovesical fistula	10.4	78	1.44	7
*Diverticulitis	20.1	68	1.34	11
*Appendicitis, peritonitis	27.5	59	1.31	10
*Gangrene of ileum	5.9	79	1.20	10
*Retroperitoneal hemorrhage; ruptured bladder; pneumonia	8.2	89	1.16	10

*Died.

TABLE III

	OBSERVATION FIRST	OBSERVATION FINAL	INTERVAL IN DAYS
Frostbite, both feet. Demarcation with loss of toes under conservative treatment. Uneventful recovery	3	1	21
Bullet wound of chest; hemothorax. Uneventful recovery	1	0	8
Perinephritic abscess. Drainage. Uneventful recovery	4	1	7
Volvulus of ileum. Resection with ileostomy. Slow convalescence	6	2	19
Incomplete abortion. Pelvic thrombophlebitis following curettage. Recovered	5	1	12
Ruptured appendix with peritonitis. Uneventful recovery	5	0	10
Nephrolithiasis. Operation followed by pneumothorax and collapse of right lung; pneumonitis. Recovered	8	2	20
Gangrenous appendix with peritonitis. Died on fourth day	5	10	4
Intestinal obstruction due to adhesions. Operation followed by aspiration pneumonia. Died	6	9	3
Multiple fractures; retroperitoneal hemorrhage; anuria. Died	4	12	4

and by "final observation" is meant the observation at the time of discharge or death. The interval in days is the interval between the two observations, but it ought to be pointed out that in actual practice observations are made daily, and sometimes more frequently. Table III illustrates the fact that an improvement in the grade corresponds to a good prognosis, while the abnormal cytological features become more prominent as the clinical condition becomes worse. This conclusion is borne out by an experience which now extends to an examination of several thousand blood films.*

CONCLUSIONS

1. In toxic conditions the polymorphs show a number of cytological changes, the principal ones observed with Giemsa stain being toxic granules, an ameboid outline, cytoplasmic vacuoles, and pyknotic areas in the nucleus. A simple method is described for grading the cells of a film according to the degree to which each of these abnormal appearances is developed, the grade varying from 0 for normal cells to 12 for the polymorphs seen in extremely toxic conditions.

2. The grade assigned by this method closely reflects the clinical condition of the patient, much more so than the total white blood cell count, the polymorph percentage, the "degenerative index" (which it replaces), or even the polynuclear count. Changes in the grade taken together with changes in the polynuclear count are of real prognostic value.

REFERENCES

Amidon, E. L.: Hematologic Studies in Acute Infections, J. Lab. & Clin. Med. 24: 1009, 1939.

Bodkin, E.: Über die Loslichkeit der weissen Blutkorperchen in Peptonlosungen, Virchows Arch. f. path. Anat. 137: 476, 1892.

Cesaris-Demil, A.: Über die morphologischen Structur mit den morphologischen und chromatischen Veraenderungen der Leukozyten, Virchows Arch. f. path. Anat. 195: 1, 1908.

Cooke, W. E., and Ponder, E.: The Polynuclear Count, London, 1927, Charles Griffin Co.

Curphey, T. J., and Ponder, E.: The Leucocytic Response in Experimental Shock, Am. J. Path. 17: 602, 1941.

Ewing, J.: Clinical Pathology of the Blood, Philadelphia, 1901, Lea Brothers and Co.

Osgood, E. E., and Ashforth, C. M.: Atlas of Hematology, San Francisco, 1937, J. W. Stacey, Inc.

Strumia, M. M.: Hematological Tables, Bryn Mawr Hosp., Philadelphia 1: 20, 1936.

*Amidon (1939) remarks that the information gained from various hematological methods of examination must be balanced against the time, effort, and equipment required, and concludes that the determination of the degenerative index, in particular, is impracticable because it requires a degree of training and technical skill not to be expected of the average hospital technician. In our experience there is no greater difficulty in grading white blood cells than there is in doing a differential white blood cell count on abnormal blood or in doing a polynuclear count. Even the beginner is not likely to have much difficulty if he once realizes that it is essential to standardize the staining method.

IS THE MAINTAINED SEDIMENTATION RATE SPECIFIC FOR MALIGNANCY?*

JULIUS J. HERTZ, M.D., AND SEYMOUR H. RINZLER, M.D.
NEW YORK, N. Y.

WITH THE TECHNICAL ASSISTANCE OF HELEN BERNSTEIN

IN 1937 Koster[1] drew attention to the fact that the sedimentation rate of erythrocytes in stored citrated blood is subject to changes; that is, that over a twenty-four-hour period the blood sedimentation rate shows a tendency to decrease. However, in certain specimens of blood the sedimentation rate remains the same throughout the twenty-four-hour period, for example, in malignant tumors and Hodgkin's disease. This observation was found to be correct in 95 per cent of 112 cases of malignant tumors and 14 cases of Hodgkin's disease. In 100 normals and 460 nonmalignant cases the rate was found to decrease steadily over a twenty-four-hour period (Table I). In 1940 Feldman[2] repeated Koster's work. His study consisted of 176 cases, of which 118 were proved malignancies and 55 were pathologic conditions other than malignancy. The percentage of positive results in the malignant cases was 95.7 per cent, and of negative results was 94.6 per cent in the nonmalignant cases. The methods of Koster and Feldman differed only in that the former used a Westergren tube and the latter used a Wintrobe tube in determining the sedimentation rate.

TABLE I (After Koster)

SEDIMENTATION RATE	DIRECT	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	6 HR.	24 HR.
Carcinoma of stomach (positive test)	17½	17	16½	17	17	19	20	17½
Ulcerative colitis (negative test)	30	27	25	26	22	16	14	3

The importance of further confirmation of such a relatively simple laboratory procedure seemed to us obvious.

METHOD

Our technique was essentially the same as described by Feldman. Two cubic centimeters of 3.8 per cent sodium citrate were placed in a dry 10 c.c. syringe and 8 c.c. of venous blood were drawn up. This mixture was used as the pooled specimen with which sedimentation rates were done directly and after one, two, three, four, and twenty-four hours. Koster and Feldman did rates for six hours the first day. We found that after four hours the tendency of the curve was established. The blood was thoroughly shaken before each

*From the Medical and Surgical Services of Beth Israel Hospital, New York City.
Received for publication, June 24, 1942.

new sedimentation rate was done so that the cells which had settled out could be thoroughly mixed with the serum. The tubes were read exactly one hour after filling. Wintrobe sedimentation tubes and a Wintrobe rack were used. Both the tube and pipettes were carefully cleaned and dried with alcohol and ether before use. The pooled specimen was placed in a refrigerator overnight, and the next day the temperature of the specimen was allowed to return to room temperature before the twenty-four-hour rate was done. Duplicate tests showed no greater variation than 2 to 3 mm. in the comparison of hourly sedimentation rates (Table II).

TABLE II

SEDIMENTATION RATE	DIRECT	1 HR.	2 HR.	3 HR.	4 HR.	24 HR.
First test	13	12	7	7	6	4
Second test	11	10	8	8	7	4

According to Koster, the criteria for a positive test (malignancy) are as follows (Table III):

TABLE III (After Koster)

SEDIMENTATION RATE	DIRECT	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	6 HR.	24 HR.
Type 1	17½	17	16½	17	17	19½	20	17½
Type 2	70	73	78	76	95	96	94	107
Type 3	30	30	35	38	39	38	36	16

Type 1: The rate of sedimentation remains at the same level throughout the twenty-four hours, or it does not vary to more than 20 per cent of the initial value.

Type 2: The rate of sedimentation markedly increases above the initial value in the course of the estimation and does not decrease to below the initial value.

Type 3: After having risen to above the initial value, the rate of sedimentation again decreases gradually, often to far below the initial value.

MATERIAL

The patients were selected from the adult medical and surgical wards of Beth Israel Hospital and from our private practice. All diagnoses of malignancy were confirmed either by roentgenogram, biopsy, surgery, or necropsy. In the majority of instances the cases were taken as consecutive admissions. In all, there were 100 patients of whom 33 had proved malignancies.

RESULTS

Our percentage accuracy for diagnosing malignancies was 54 per cent (Table IV) as compared with Koster's 95 per cent and Feldman's 95.7 per cent (Table V). Our percentage accuracy in nonmalignant cases was 72 per cent as compared with Koster's 100 per cent and Feldman's 94.6 per cent. Sedimentation tests were repeated on the majority of our false negatives two or more times.

DISCUSSION

Our results obviously do not agree with those of either Koster or Feldman. In our series of malignant cases about one-half gave false negatives. False

negatives may be attributed to hemolysis of the specimen, insufficient shaking before each sedimentation rate so that the cells and serum are not thoroughly mixed, or not allowing the twenty-four-hour specimen to come to room temperature after removing it from the refrigerator. All of these make for a slower sedimentation rate and, therefore, tend toward the negative curve. These pitfalls were carefully avoided, as shown by the fact that ten patients on whom tests were done at the same time from specimens taken by two injections showed no more than 2 to 3 mm. variation in the hourly specimens (Table II).

TABLE IV

	TOTAL NO.	WITH POSITIVE SEDIMENTATION RATE	WITH NEGATIVE SEDIMENTATION RATE	PERCENTAGE ACCURACY
Proved malignancies	33	18	15	54
Nonmalignant	67	49	18	72

TABLE V

	% POSITIVE	% NEGATIVE
Koster	95	100
Feldman	95.7	94.6
Hertz and Rinzler	54	72

According to Koster and Feldman, false positives may result from the following drugs: the sulfonamides, potassium iodide, bismuth, salvarsan, aminopyrine. We have eliminated from this series any patients who were taking any of the sulfonamides. Other drugs taken by our patients include: atropine, phenobarbital, thyroid extract, thiamine chloride, caffeine citrate, ammonium chloride, amytal, cevitamic acid, feosol, and Lugol's solution. These drugs were taken in therapeutic doses by both the true negatives and false positives, both groups being nonmalignant. Diagnoses on some of the false positives were: chronic diffuse glomerular nephritis (3 tests); calculus of the common duct (5 tests); congestive heart failure (2 tests); and lupus erythematosis (2 tests).

We, therefore, feel that this test is of no value in our experience in accurately differentiating malignant from nonmalignant disease.

SUMMARY

Sedimentation tests done serially according to the methods of Koster and Feldman on 33 patients with proved malignancies and 67 patients with pathologic conditions other than malignancy showed 54 per cent positive results in the malignant cases and 72 per cent negative results in the nonmalignant cases. We must conclude that in our hands the test is not accurate for use in the differential diagnosis between malignant and nonmalignant conditions.

REFERENCES

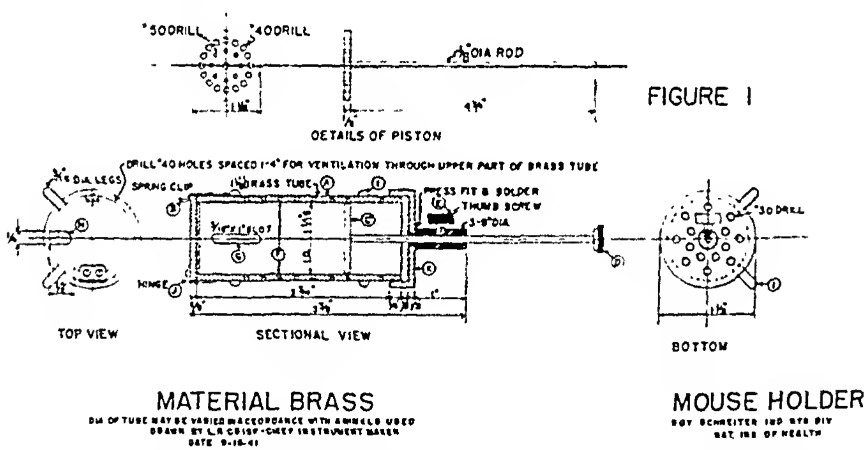
1. Koster, L.: Changes in Sedimentation Rate of Erythrocytes in Stored Citrated Blood as an Aid to Diagnosis in Cases of Malignant Tumours and Lymphogranuloma, Acta med. Scandinav. 93: 420, 1937.
2. Feldman, H.: Maintenance of the Sedimentation Rate of Erythrocytes in Vitro in Cases of Malignant Tumors and Hodgkin's Disease, Am. J. M. Sc. 200: 820, 1940.

MOUSE HOLDERS FACILITATING INTRAVENOUS AND INTRAPERITONEAL INJECTIONS*

Roy Schneiter, M.S., and Lawrence R. Crisp

Although the intravenous injection of mice is a relatively simple procedure, the use of this route of inoculation is often limited because of the lack of a satisfactory apparatus for holding the animal during injection.

Very few satisfactory mouse or rat holders have ever been described. Burdon[1] described a mouse holder which consists essentially of a slitted, illuminated box on top of which is attached a wire cage for holding the animal. This holder, although it has many desirable features, appears to be somewhat cumbersome, and it is not adjustable to the length of the animal.



The apparatus described in this report was designed for simplicity and rapidity of operation and is adjustable to the length of the animal. Two types of holders have been designed (Figs. 1 and 2). The holders illustrated are made of brass tubing of suitable diameter for accommodating either large or small mice. The tube has a hinged, slotted door at the end or is hinged at the long, horizontal axis of the tube to permit the admission of the animal. A plunger for adjusting the length of the tube to the length of the animal is provided, and the animal is thus immobilized for either injection or bleeding. These holders are compact and easy to use and may be completely dismantled for cleaning and sterilization. With very little practice, mice may be readily induced to enter the holder.

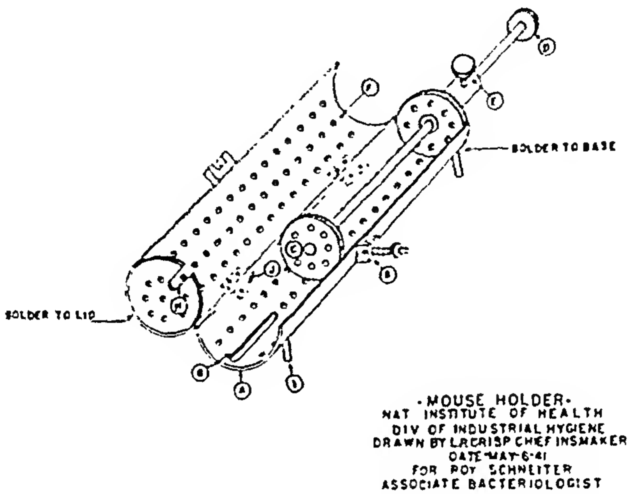
*From the Division of Industrial Hygiene, National Institute of Health, United States Public Health Service, Bethesda, Md.

Received for publication, May 23, 1942.

By a selection of brass tubing of suitable diameter, either type of holder may be constructed for work with rats.

Each type of holder has been designed with a slit to permit intraperitoneal injections. A single worker with the aid of these holders can readily make intravenous or intraperitoneal injections without assistance.

FIGURE 2



Diagrammatic sketches of the two types of holders are given in Figs. 1 and 2. The essential parts of the holders are described as follows: *A*, Brass tube 5 inches long, 1⅛ inches outside diameter, and ¹⁄₁₆ inch wall; *B*, spring or swinging latches; *C*, plunger for adjusting holder to the length of the mouse; *D*, handle and rod for control of plunger; *E*, set screw for locking plunger rod after adjustment; *F*, air holes Nos. 30, 40, 44, and 50 drills; *G*, slit for intraperitoneal injections; *H*, slot in end of tube for accommodating tail of animal; *I*, legs for support of holder; *J*, hinges; and *K*, threaded cap for cleaning the end-opening holder.

In making intravenous injections the holders may be placed on the laboratory table top or mounted in a ring stand. The animal is placed in the holder, and the plunger is adjusted to restrict movement. Tail veins may be brought into sharp relief by the application of xylol or alcohol. One-fourth, 0.5, or 1 c.c. hypodermic syringes fitted with 1 inch, No. 26 gauge needles are the most suitable for intravenous injections. The needle is most easily inserted at an angle almost parallel to the tail. We have found 0.25 c.c. is the maximum quantity of inoculum which can ordinarily be injected into the tail vein of the average mouse.

SUMMARY

Two types of holders have been described which facilitate the intravenous and intraperitoneal injection of mice and rats. These holders permit the utilization of these techniques by the individual worker, thus increasing the potential usefulness of these laboratory animals.

REFERENCE

1. Burdon, Kenneth L.: A Mouse Holder Facilitating Intravenous Inoculations, J. Lab. & Clin. Med. 23: 1293, 1938.

SPUTUM CONCENTRATION FOR THE CULTURE OF TUBERCLE BACILLUS*

M. Gerundo, M.D., Topeka, Kan.

Direct cultivation of tubercle bacillus has not yet become a routine procedure because of drawbacks in the manipulations and concentration of sputum.

The most widely used method is perhaps that with sulfuric acid. The sputum is mixed with equal amounts of 15 per cent sulfuric acid and then, is washed and centrifuged.

This method, however, has some drawbacks because it fails usually with dense and viscous sputa, containing large amounts of mucus and albumin. Large flakes of mucus escape contact with the sulfuric acid and finally cause contaminations of the cultures.

To obviate this difficulty, I have found the following method more satisfactory and also more reliable, because it produces a complete fluidification of the sputum, thus lessening the danger of contaminations.

The formula I use is a modified Jousset's liquid for inoscopy:

Pepsin	1 Gm.
Glycerin	10 c.c.
Hydrochloric acid	15 c.c.
Sodium fluoride	1 Gm.
Distilled water	1,000 c.c.

Mix equal volumes of sputum and solution and leave the mixture for four hours in the incubator at 37° C., shaking occasionally to facilitate homogenization.

At the end of this period centrifuge, wash with sterile saline several times to remove the acid, and make cultures with the sediment upon Petragnani's or Loewenstein's media, or prepare for microscopic examination.

The results are definitely superior to the sulfuric acid method. The culture method is preferable to animal inoculation, because it is less expensive, more reliable, and more rapid.

This method can be applied satisfactorily also to urine containing blood or albumin.

*From the Topeka State Hospital.

CHEMICAL

THE NATURE OF THE BLOOD IODINE AND ITS DETERMINATION*

SOLOMON SILVER, M.D., NEW YORK, N. Y.

HISTORICAL ASPECTS

SINCE the modest six-page contribution by Courtois[1] in 1813 in which he announced "the discovery of a new substance in seaweed," the study of iodine and its place in animal physiology has assumed ever-increasing interest and importance. In 1900 Gley and Bourcet[2] established the presence of iodine in the circulating blood and published figures that agree very closely with those accepted as normal today. The extent of these studies was limited by the fact that they required a liter of blood to complete a single analysis. In 1920 Kendall[3] reported values of about 13 gammas per 100 c.c. for whole blood in a small series. The first series of any size and the first values attempting to correlate the blood iodine level with clinical disorders of the thyroid gland was published by Veil and Sturm[4] in 1925. They reported values of 13 to 17 gammas per 100 c.c. as normal, and they found the blood iodine level to be increased in Graves' disease where their values varied from 21 to 70 gammas per 100 c.c. Veil and Sturm extended von Fellenberg's concept, which was widely and perhaps uncritically accepted, that the iodine in the blood existed in at least two forms. One form, which they called "organic," was insoluble in cold alcohol, and another, soluble in cold alcohol, they called "inorganic." In normal blood they found an average of 9 gammas per 100 c.c. "inorganic" and 5 gammas per 100 c.c. "organic" iodine. In Graves' disease they found that the increase was almost entirely in the "organic" fraction. In 1929 Lunde, Closs, and Pedersen[5] objected to the use of cold alcohol to separate the two forms of the blood iodine because, as they said, the "inorganic" component could never be extracted completely with cold alcohol and that further washings always contained iodine. They, therefore, proposed the use of hot alcohol in a Soxhlet apparatus using four volumes of alcohol to one volume of blood and extracting once for four hours. By this method the authors greatly increased the alcohol soluble or "inorganic" fraction so that normally they found from 7 to 12 gammas per 100 c.c. as "inorganic" and only 1 to 4 gammas per 100 c.c. as "organic" or remaining in the residue. Ultrafiltration was used as a means of separating the organic and inorganic fractions by Leipert[6] in 1937 and Trevorrow[7] in 1939, but their results are in conflict. The former found that from 70 to 90 per cent of the serum iodine was ultrafiltrable, whereas the latter found that absolutely no iodine passed through the ultra-

*From the Laboratories and the Medical Service of Dr. Eli Moschcowitz, Mount Sinai Hospital, New York.

Received for publication, February 25, 1942.

filter. More recently, Salter[8] has resorted to protein precipitation by heat and dilute acid and differentiates between the iodine fixed to the coagulum and that which can be recovered in the filtrate. He refers to the former as "protein" iodine and to the latter as "inorganic." He admits, however, that his cannot be used if iodides have been administered and that his method gives false values for the "protein iodine" fraction if iodide is added to plasma in vitro. It is obvious that methods which fail to eliminate ingested iodide have markedly restricted application.

The purpose of this study is to demonstrate the absence of true inorganic iodine in normal blood and to show that all the normally occurring iodine is bound to the plasma proteins. In addition, a method (dialysis) is presented which permits the determination of "protein bound" or nondialyzable iodine in the presence of inorganic iodine.

METHODS

The analyses were carried out by the Matthews[9] modification of the Leipert method with the following minor variations:

1. The final oxidation to iodate was carried out in a small volume, using bromine vapor after the solution had been rendered just acid to methyl orange with phosphoric acid. Bromine was removed by boiling.
2. N/1,000 thiosulfate was used instead of N/5,000.
3. Sulfite was not used in the receiver.

This method is accurate to 5 per cent in the range of these experiments and recoveries of added iodine compounds are complete.

Duplicate determinations were done in every case. If the results did not check, new sets of duplicate determinations were set up.

The blank for the method is less than 0.05 gamma iodine per determination.

Dialyses were carried out in cellophane casings made by the Visking Company, and the duration of each dialysis was seventy-two hours in running water. Tap water can be used for dialysis if suitable correction is made for the iodine content of the water which enters the sac during dialysis.

EXPERIMENTAL

The entire question of "organic" and "inorganic" iodine in normal blood was reopened by Trevorrow, who reported that she could extract all the iodine in normal blood by ethyl alcohol. If this is true, it is obvious that there can be no distinction between "inorganic" and "organic" fractions based upon alcohol solubility. We have been able to confirm her studies and have found that all the blood iodine can be extracted with either hot or cold alcohol, provided enough of the solvent is used and the extraction is carried on long enough. The extractions with cold alcohol were carried out in stoppered flasks in a shaking machine, and those with hot alcohol were carried out in a flask under a reflux condenser and also in a Soxhlet apparatus adding the blood to the extraction thimble after the proteins had been precipitated by the alcohol.

It is clear from these experiments and the studies of Trevorrow that no reliance can be placed upon so-called "organic" fractions of blood iodine based upon alcohol solubility. It is probable that individual workers have

TABLE I

SOLUBILITY OF NORMAL BLOOD IODINE IN COLD AND BOILING ALCOHOL
Iodine Content of Residue (in gamma)

(In all experiments 50 c.c. of whole blood containing 1.8 gamma of iodine were extracted with 400 c.c. of alcohol and each extraction lasted twenty-four hours)

	COLD ALCOHOL	BOILING ALCOHOL	SOXHLET APPARATUS
After one extraction	0.8	0.6	0.5
After two extractions	0.4	0.3	0.2
After three extractions	<0.1	<0.1	<0.1

TABLE II

BLOOD IODINE VALUES BEFORE AND AFTER DIALYSIS
(In gamma per 100 c.c.)

SOURCE OF BLOOD	IODINE CONTENT BEFORE DIALYSIS (AVERAGE)	IODINE CONTENT AFTER DIALYSIS (AVERAGE)
Sheep's blood		
Lot 1	3.2	3.4
Lot 2	3.0	3.0
Lot 3	2.0	2.0
Lot 4	2.1	2.4
Lot 5	3.4	3.0
Lot 6	1.9	1.9
Average	2.6	2.6
Human blood controls		
1	3.2	3.0
2	3.6	3.0
3	2.6	2.9
4	2.0	2.1
5	2.4	2.3
6	3.0	3.0
7	4.0	3.9
8	3.5	3.7
9	3.4	3.4
10	4.1	4.0
Average	3.2	3.1
Human blood Graves' disease		
1	8.6	8.0
2	10.2	10.6
3	13.0	13.0
4	12.0	11.5
5	7.5	7.5
6	8.0	8.1
7	9.7	10.1
8	6.8	6.4
Average	9.6	9.4

TABLE III

IODINE CONTENT OF WASHED RED BLOOD CELLS

VOLUME OF PACKED RED BLOOD CELLS (C.C.)	IODINE CONTENT IN GAMMA PER 100 C.C.
5	<0.1
10	<0.1
15	<0.1
25	<0.1

obtained relatively constant fractions because they kept the conditions of their extractions constant and, therefore, extracted a fairly constant proportion of the total blood iodine. This may also explain the variations in the ratio of "inorganic" to "organic" iodine from 3 to 1, as reported by Lunde and Closs[5] (or even 5 to 1) to 2 to 3, as reported by Perkin.[10] It is significant that the authors who obtained the highest "inorganic" fractions used hot alcohol in their extraction procedures.

It was clear to us that we could not use alcohol as a solvent to obtain significant partition values for the blood iodine or to differentiate between normal circulating iodine and ingested iodine. It occurred to us, as it had to Gley and Bourcet forty years ago, that the normally circulating iodine might be bound to protein and, therefore, resistant to dialysis. This proved to be the case as is shown in Table II.

It can be seen from these experiments that all the iodine that circulates normally or in Graves' disease is in a form that resists dialysis and is, therefore, fixed to a large molecule, probably protein.

TABLE IV

DIALYSIS OF WHOLE HUMAN BLOOD TO WHICH POTASSIUM IODIDE HAS BEEN ADDED IN VITRO

(In each experiment 25 c.c. of whole blood containing 0.8 gamma of iodine was used)

AMOUNT OF POTASSIUM IODIDE ADDED (γ)	TOTAL IODINE CONTENT AFTER 72 HOURS' DIALYSIS (γ)	AVERAGE CHANGE IN IODINE CONTENT (γ)
5	0.9	-0.2
	0.8	
	0.8	
	0.6	
10	0.8	0
	0.7	
	0.8	
	0.9	
50	1.0	-0.2
	0.8	
	0.6	
	0.7	
100	0.8	-0.2
	0.8	
	0.7	
	0.8	
1,000	1.1	+0.2
	0.7	
	0.8	
	0.7	
5,000	0.7	0
	0.8	
	0.9	
	0.8	

DISTRIBUTION OF BLOOD IODINE IN CELLS AND PLASMA

There is no agreement in the literature regarding the proportion of the normally circulating blood iodine that exists in the red blood cells as compared with the concentration in the plasma. In 1900 Gley and Bourcet said that the red blood cells contained no iodine whatsoever. In 1940 Salter[11] indicated that

he thought there was as much iodine in the cells as in the plasma; he stated that in a euthyroid person the total blood iodine was 6 gammas per 100 c.c., the plasma iodine was 6 gammas per 100 c.c., and the iodine contained in the red blood cells was also 6 gammas per 100 c.c. Such values imply equal iodine distribution between plasma and cells when the hematocrit reading is 50 per cent. Trevorrow stated that "whole blood and plasma showed a distribution of the total iodine in proportion to the water content of the plasma and cells." Klassen, Bierbaum, and Curtis[12] found that the red blood cells account for only 5 per cent of the blood iodine and even this must be viewed with some reserve because the methods used have an error as large as 5 per cent.

TABLE V

BLOOD IODINE VALUES

(Values expressed in gamma per 100 c.c. when one gram of potassium iodide is administered by mouth three times daily for three days to normal adults)

CASE	CONTROL PERIOD		AFTER INGESTION OF POTASSIUM IODIDE	
	TOTAL BLOOD IODINE	NONDIALYZABLE BLOOD IODINE	TOTAL BLOOD IODINE	NONDIALYZABLE BLOOD IODINE
1	4.5	4.0	825	4.0
2	4.4	4.3	385	4.0
3	3.6	3.6	624	3.9
4	4.3	4.1	465	4.3
5	3.8	3.6	726	4.1
6	3.4	3.4	1,290	3.4
Average		3.8		3.9

TABLE VI

DIALYSIS OF WHOLE BLOOD TO WHICH THYROXINE AND l-DIIODOTYROSINE (IN AQUEOUS SOLUTION) HAVE BEEN ADDED

VOLUME OF BLOOD (C.C.)	IODINE CONTENT OF ADDED THYROXINE (γ)	THYROXINE RENDERED NONDIALYZABLE (%)
5	2	46
5	5	40
5	20	40
5	50	34
25	2	68
25	5	60
25	20	48
25	50	40
VOLUME OF BLOOD (C.C.)	IODINE CONTENT OF ADDED DIIODOTYROSINE (γ)	DIIODOTYROSINE RENDERED NONDIALYZABLE (%)
5	2	17
5	5	12
5	20	8
5	50	5
25	2	35
25	5	28
25	20	17
25	50	11

Our studies with red blood cells washed four times with iodine-free 0.9 per cent sodium chloride solution reveal only traces of iodine. In our opinion the red blood cells are free of any significant quantity of iodine detectable by present methods. This is easily understood because all the blood iodine is in the plasma in a nondiffusible form, and the surface of the red blood cells probably acts as a semipermeable membrane, preventing the iodized molecule from entering.

It remained for us to demonstrate that iodide added to blood in vitro or ingested in vivo could be differentiated from the normally circulating blood iodine. For this separation the procedure of dialysis again proved completely satisfactory.

The representative data in tables demonstrate that the procedure of dialysis is a satisfactory answer to the problem of differentiating between normally circulating blood iodine and iodine ingested in the form of iodide.

In the course of these studies it became evident that certain organic iodine compounds reacted in a manner very different from the behavior of the inorganic salts of iodine. When these compounds were added to whole blood, plasma, or other protein solutions, they could not be removed quantitatively by dialysis, although they are easily dialyzable in aqueous solution. Apparently new compounds were formed with the proteins and these compounds resisted dialysis. A large series of organic iodine compounds was studied using different protein solutions. The quantitative aspects of the reaction are not simple, but the proportion of the added iodine compound rendered nondialyzable varied with the protein, the organically bound iodine compound, and the relative amounts of each employed. The following substances behaved in a qualitatively similar fashion: thyroxine, diiodotyrosine, tetraiodophenolphthalein, diodrast, and hippuran.

SUMMARY

1. All the normally circulating blood iodine can be extracted with ethyl alcohol if proper precautions are taken. The distinction between organic and inorganic blood iodine based upon alcohol solubility is without foundation, and significant quantities of "inorganic" iodine in normal blood exist only as an artifact of chemical manipulations.

2. Practically all the normally circulating blood iodine is in the plasma. The amount present in washed red blood cells is too low to be determined by present methods.

3. All the normally circulating blood iodine or that circulating in Graves' disease is in a form which resists dialysis and is probably bound to protein.

4. Dialysis removed from the blood all iodine added or ingested in the form of iodide and can be used to remove ingested iodide known to the observer or unsuspected by him.

5. Dialysis does not remove completely added or ingested iodine in the form of diiodotyrosine or thyroxine.

REFERENCES

1. Courtois, B.: Découverte d'une substance nouvelle dans le vereeh, Ann. d. Chem. 88: 304, 1813.
2. Gley, E., and Bourcet, P.: Présence de l'iode dans le sang, Compt. rend. Acad. d. sc. 130: 1721, 1900.
3. Kendall, E. C., and Richardson, F. S.: Determination of Iodine in Blood and in Animal Tissues, J. Biol. Chem. 43: 161, 1920.
4. Veil, W. H., and Sturm, A.: Beiträge zur Kenntnis des Jodstoffwechsels, Deutsch. Arch. f. klin. Med. 147: 166, 1925.
5. Lunde, G., Closs, K., and Pedersen, O. C.: Untersuchungen über den Jodstoffwechsel, Biochem. Ztschr. 206: 261, 1929.
6. Leipert, T.: Zur Kenntnis des Blutjods, Biochem. Ztschr. 293: 99, 1937.

7. Trevorrow, V.: Studies of the Nature of the Iodine in the Blood, J. Biol. Chem. 127: 737, 1939.
8. Salter, T. H.: The Endocrine Function of Iodine, Cambridge, Mass., 1940, page 75.
9. Matthews, N. L., et al.: Determination of Iodine in Biological Materials, Indust. & Engin. Chem. 10: 612, 1938.
10. Perkin, H. J., and Hurxthal, L. M.: The Fractionation of the Iodine of the Blood in Thyroid Disease, J. Clin. Investigation 18: 733, 1939.
11. Salter, T. H.: The Endocrine Function of Iodine, Cambridge, Mass., 1940, page 76.
12. Klassen, K. P., Bierbaum, R. L., and Curtis, G. M.: The Comparative Iodine Content of Whole Blood and Serum, J. Lab. & Clin. Med. 26: 365, 1940.

Acknowledgment is gratefully made to Roche-Organon, Inc., for their generous aid in supplying the thyroxine and diiodotyrosine used in the basic studies.

UREA SPOT TEST FOR THE DETECTION OF NITROGEN RETENTION*

Hans Norbert Naumann, M.D., Milton Plotz, M.D., F.A.C.P., and Nathaniel E. Reich, M.D., Brooklyn, N. Y.

In 1922 Weltmann and Barrenscheen[5] called attention to the yellow reaction of urea with Ehrlich's para-dimethylaminobenzaldehyde reagent and suggested its use as a simple clinical test for nitrogen retention. This test was recommended some years later by Hunt.[3] In 1934 Patterson[7] described a spot test based on Weltmann and Barrenscheen's color reaction. The test was performed by mixing a few drops of blood with an Ehrlich reagent containing trichloroacetic acid in a small test tube and pouring the mixture on filter paper. A yellowish coloration of the moist area around the dark brown protein precipitate in the center indicated a substantial increase of blood urea. Krieger in 1934[4] confirmed the reliability of the test and introduced color standards of picric acid on filter paper. However, Heggie[2] in 1938. after investigating Patterson's spot test with blood specimens of known urea concentration, arrived at the conclusion that the method is inadequate not only for high concentrations, but also for the most important clinical range of 50 to 100 mg. per 100 c.c. urea. The potential usefulness of such a test as an emergency bedside procedure, if reliable, stimulated the present investigation and led to a satisfactory modification of the original Patterson test.

We agree with Heggie in concluding that Patterson's urea spot test in its original form is not adequate because under the conditions of the test even a considerable elevation of blood urea cannot be detected with any degree of accuracy. As long as the spot on the filter paper is still wet, it is impossible to see a definite yellow coloration, except perhaps in very high concentrations. In the course of drying the yellow color becomes gradually more distinct, but so does the color of the reagent itself. It is difficult, therefore, to note whether the yellow color is due to the urea reaction or to the color of the reagent.

*From the Department of Medicine, Long Island College of Medicine, and the Kings County Hospital (Long Island College of Medicine Division), service of Dr. Carl H. Greene.
Received for publication, May 22, 1942.

From these findings two immediate objectives of our investigation were clearly indicated:

1. Inasmuch as the yellow color reaction becomes distinct after drying, a method of drying had to be found which would give a clear end point.
2. The error due to the yellow color of the reagent had to be eliminated.

MODIFICATION OF THE UREA SPOT TEST

1. *Drying.* Experiments showed that it is not necessary to dry the filter paper for many hours (as advocated by Patterson and Krieger). It may be dried conveniently over any source of heat, provided the heat is applied equally to the entire spot. It is essential not to have the spot in direct contact with the source of heat, but at some distance from it. A bridge or desk lamp may be used as the source of heat or, better still, the filter paper may be allowed to rest on a steam radiator for several minutes while the doctor or technician is free to engage in other work.

2. *Elimination of yellow color of the reagent.* On the assumption that the yellow color of the reagent might be due to various degrees of purity, different preparations of p-dimethylaminobenzaldehyde were examined.* The Eastman drug gives a slightly more intense, yellow coloration than the English preparation. Therefore, only the Eastman p-dimethylaminobenzaldehyde was used.

It was found, in addition, that the solvents and the method of preparation are of importance. For example, alcoholic solutions increase the yellow color of the reagent considerably. It was also important not to use an excess of the reagent. The reagent finally was prepared as follows:

P-dimethylaminobenzaldehyde "Kodak"	0.5 Gm.
Trichloroacetic acid	100.0 Gm.
Distilled water up to	100.0 c.c.

The reagent should be kept for convenience in a dropper bottle.

The p-dimethylaminobenzaldehyde dissolves easily in a concentrated trichloroacetic acid, producing a slightly yellow color. A drop of this reagent on filter paper dried for ten to fifteen minutes on a hot surface gives a pinkish to grayish spot, contrasting satisfactorily with the yellow reaction of the urea. Only on prolonged heating or after the paper is kept for many hours does the spot of dry reagent turn yellow.

In order to eliminate further any possibility of error due to the coloration of the reagent, it was found expedient to place the blood to be tested in the middle of a wet spot of reagent instead of mixing blood and reagent before applying the mixture to filter paper. This technique is sufficient to precipitate most of the blood proteins in a small drop and allows a clear differentiation between the yellow zone surrounding the dark brown spot of blood proteins from the outer pinkish to grayish zone of reagent itself. In addition, two further advantages exist. There is no necessity of using a small test tube for

*These samples were marketed by the following firms: British Drug Houses Ltd., London. Eastman Kodak Co., Inc., Rochester, N. Y. Eimer & Amend, New York. Hartman-Leddon Co., Philadelphia.

We wish to express our appreciation to the Eastman Kodak Co. for giving us a sample of their preparation.

mixing blood and reagent, and the amount of blood necessary for the test is decreased from several large drops to a single small drop, making this method a true microtechnique.

A drop of reagent is dropped on a small piece of filter paper. The finger is punctured in the usual manner for obtaining blood counts, and a small drop is allowed to come into contact with the moist area of the paper. The paper is dried, as described before, and is read after several minutes. A small spot of dark brown coagulated blood protein is seen in the center surrounded by a narrow yellow zone produced by the urea reaction and a pinkish-gray outer zone caused by the color of the reagent itself. The test is improved greatly in accuracy by turning the paper over and reading the result on the opposite side.* The intensity of the color reaction may be graded from 0 to 4 plus. It is wise at first to obtain a control reaction with normal blood, but those who have worked with this test soon found that a control was unnecessary. If the test is used infrequently, it is well to use a control.

INTERPRETATION OF THE TEST

The test as described is sensitive enough to show a weak yellowish zone in normal blood. Comparatively small increases of urea nitrogen up to 25 mg. per 100 c.c. (about 50 mg. per 100 c.c. urea) can be detected by comparison with the reaction of normal blood, provided the conditions of the test (amount of wet reagent, surface of blood in contact with liquid reagent, and equal heating of both spots) are kept reasonably constant. The test is not a quantitative one except very roughly. Twenty to 40 mg. per 100 c.c. of urea nitrogen produced a yellow color slightly more intense than normal; 40 to 60 mg. per 100 c.c. gives a moderately intense reaction; above 60 mg. per 100 c.c. the color is very intense.

The only compounds in blood which might interfere with the urea reaction are the sulfonamides. These dyes can be considered as derivatives of sulfanilic acid which was listed as an interfering substance with Ehrlich's reagent by one of the authors (Naumann, 1938). Monto,[5] and more recently, Churg and Lehr[1] described a quantitative determination of sulfonamides in blood based on the yellow color reaction with Ehrlich's reagent. The urea spot test as described may also be used for the detection of the sulfa drugs in blood if only a rough estimate is required. Further investigation along these lines is now under way. While conditions with marked nitrogen retention will as a rule not be those in which sulfonamide treatment may be expected, it is well to keep this possibility in mind in those cases where treatment prior to the test is unknown.

SUMMARY

1. A simple urea spot test for the detection of nitrogen retention is described, based on the yellow color reaction of urea with Ehrlich's aldehyde reagent.

2. The test, requiring a specially prepared reagent, filter paper, and a heat source, can be completed in ten minutes.

3. The sensitivity of the test is sufficient to detect slight, moderate, and marked increase of blood urea.

*Noted by Dr. Kwascevska.

4. The urea spot test may also be used for the detection and rough estimation of sulfonamides in blood which give a similar yellow color reaction. The possible interference of urea and sulfonamides with each other is pointed out.

REFERENCES

1. Churg, Jacob, and Lehr, David: Determination of Sulfanilamide and Its Derivatives in Blood, Am. J. Clin. Path. (Tech. Sect.) 6: 22, 1942.
2. Heggie, R. M.: Observations on the Use of Ehrlich's Reagent in the Estimation of Blood Urea, J. Roy. Nav. M. Serv. 24: 198, 1938.
3. Hunt, T. C.: Recent Aids in the Diagnosis of Common Disorders, Lancet Oct. 12, p. 753, 1929.
4. Krieger, V. I.: A Simple Colorimetric Method of Determining the Concentration of Urea in Blood, M. J. Australia Sept. 14, p. 340, 1935.
5. Monto, Raymond W.: The Determination of Sulfanilamide and Related Compounds in Body Fluids by a Simple Rapid Method, Am. J. Clin. Path. 11: 165, 1941.
6. Naumann, Hans N.: The Reaction of Urobilinogen With p-Dimethylaminobenzaldehyde, J. Lab. & Clin. Med. 23: 1127, 1938.
7. Patterson, Jocelyn: Chemical Spot Test in Diagnosis of Uremia, Lancet 1: 1061, 1934.
8. Weltmann, O., and Barrenscheen, H. K.: Ueber eine neue Harnstoffreaktion und ihre klinische Verwertbarkeit, Klin. Wchnschr. 1: 1100, 1922.

THE TRYPTOPHAN TEST IN GENERAL PARESIS*

M. J. Madonick, M.D., and J. Lissman, M.D.
New York, N. Y.

WE BECAME interested in the tryptophan test in general paresis as a result of our experience with a case of this disease on the wards of the Morrisania City Hospital in which the reaction was positive. A further evaluation of this test in syphilis was then undertaken and it was performed on the spinal fluids of 133 paretic persons. Cases were classified as paresis in which positive blood and spinal serology and the mental picture of paresis were all present. Because of the clinical features and positive serology both in blood and spinal fluid, three cases which did not have the typical paretic gold curve were considered as cases of paresis. In addition, the spinal fluids of several cases of taboparesis, latent syphilis, meningovascular or cerebral syphilis, and nonneurological syphilis, were studied. As a control series the fluids of 102 cases of alcoholism, the major psychoses and general medical conditions were examined. Fluids from patients with a history of syphilis or with a positive serology were excluded from this control group.

The technique of the test employed was that described by Lichtenberg[19] and Aiello.[1] It is essential that the spinal fluid be clear; we found, as have other workers,[2, 4, 19, 28] that xanthochromic, hemorrhagic, and purulent fluids gave "false positive" reactions.

Table I indicates the distribution of our cases. One hundred and nine, or 82 per cent, of the 133 paretic fluids gave a positive reaction. In the control

*From the Neuropsychiatric Service of the Morrisania City Hospital, N. Savitsky, M.D., chief, and the Psychiatric Division of Bellevue Hospital, New York City, C. N. Colbert, M.D., acting director.

Received for publication, June 4, 1942.

series only two positive responses were obtained; one was in a case of cerebral arteriosclerosis and one in a mild rheumatic fever. We had three persons with multiple sclerosis with paretic gold curves who gave negative responses.

TABLE I

DIAGNOSIS	TOTAL NO. CASES	GOLD CURVE	NO. CASES	TRYPTOPHAN POSITIVE	TRYPTOPHAN NEGATIVE	PER CENT POSITIVE
Paresis	133	Paretic	130	106	24	82
		Flat	1	1	0	
		Meningitic	2	2	0	
Taboparesis	3	Paretic	1	1	0	
		Low paretic	1	1	0	
		Syphilitic	1	1	0	
Tabes	4	Syphilitic	4	0	0	0
Asymptomatic neurosyphilis	11	Flat	11	0	11	0
Psychosis with cerebral or meningovascular syphilis	13	Paretic	2	2	0	46.1
		Syphilitic	4	0	4	
		Flat	7	3	4	
Syphilis with positive blood and negative spinal serology	11	Flat	11	0	11	0
Controls	102	Flat	102	2	100	2
Multiple sclerosis	3	Paretic	3	0	3	0
Psychosis with organic brain disease	1	Syphilitic	1	0	1	0

The positive response in the cerebrospinal fluid to the tryptophan test depends upon the presence of the amino acid with the same name. Riebeling[24] states that no tryptophan is found in the normal spinal fluid. This substance, according to Mouriz,[21] is normally present in the cerebrospinal fluid up to 1 mg. per 100 c.c. Jequier and Roten[17] by adding tryptophan to normal spinal fluids which gave no response to the test found that if there is less than 0.0001 Gm. of this substance in the testing fluid the result is negative. At this level a faint ring is seen. Brugi[7] found that one part of tryptophan to 10,000 parts of diluent resulted in a positive reaction.

Boltz[6] studied the spinal fluid by the test which bears his name. Grossman[13] and Harris[15] believed the test was due to cholesterol, although Boltz was uncertain as to the chemical basis for the reaction. Later authors, Riebeling, Blix and Backlin[3] and Walker and Sleeper,[31] state that this is a test for tryptophan. Table II indicates the results obtained by Boltz and other investigators employing his test or modification of it.

In the report of Steele and Nicole,[29] six of the positive cases had a syphilitic gold curve; the remainder, including the negative responses, were in cases with a paretic gold curve. They stated that 8 per cent of neurosyphilitics give a positive result, although they are not true cases of paresis. Greenfield and Carmichael[11] were of the opinion that there was no relationship between changes in the colloidal gold curve and the test. All Thomas'[30] cases of paresis had a paretic gold curve.

TABLE II

TEST	AUTHOR	CASES	TRACES	POSITIVE	NEGATIVE	PER CENT POSITIVE
Boltz	Boltz[6]	22 paresis		22	0	100
		2 taboparesis		1	1	50
		6 tabes		0	6	0
		10 psychosis with cerebral arteriosclerosis		3	7	30
	Grossman[13]	28 paresis		28	0	100
		1 tabes		0	1	0
		31 other diseases		0	31	0
	Duncan[9]	27 paresis		27	0	100
		127 other mental conditions		121	6	94
	Harris[15]	92 paresis		89	3	97
		5 neurosyphilis		2	3	40
		83 other mental conditions		1	82	1
	Steele and Nicole[29]	36 paresis		35	1	97
	Greenfield and Carmichael[11]	16 paresis		15	1	94
		6 tabes		0	6	0
		4 cerebral syphilis		0	4	0
	Schreus and Willms[26]	11 paresis or taboparesis		11	0	100
		24 tabes		15	9	62
	Thomas[30]	33 paresis		23	10	70
		1 tabes		0	1	0
	Piotrowski[22]	10 paresis		6	4	60
		5 tabes		0	5	0
		10 cerebral degenerative disorders		7	3	70
Riebeling	Riebeling[24]	73 paresis		68	5	93
Kraus and Mezey colorimetric method	Kraus and Mezey[18]	11 paresis	5	5	1	
		3 taboparesis	1	1	1	
		6 tabes	2	1	3	
		7 cerebrospinal syphilis	3	2	2	
		5 latent syphilis	1	3	1	
		1 gumma brain	0	1	0	
Aiello	Aiello[1]	7 paresis		0	7	0
		9 tabes		0	9	0
	Spillane[28]	8 paresis		0	8	0
		4 cerebral syphilis		0	4	0
	Baxter[2]	3 paresis		0	3	0

A positive tryptophan test has been considered by many writers to be specific for tuberculous meningitis.[1, 5, 8, 12, 14, 19, 23, 25, 27, 28] Spillane,[28] accepting the work of Fildes and Knight,[10] on the ability of the tubercle bacilli to synthesize their own tryptophan in vitro on media lacking this amino acid, believes that a positive test is, therefore, due to the presence of the tubercle bacillus. Boltz and Grossman,[13] interested in this test in syphilis, felt that it was an index of the destruction by the *Treponema pallidum* upon the tissues of the central nervous system. Massazza[20] thought that it was due only to the breakdown of protein. Mouriz and Piotrowski[22] believed that tryptophan was

increased in processes in which there is destruction of cells in the central nervous system. A similar view is offered by Kraus and Mezey.[15] The last-mentioned authors obtained a positive tryptophan test only in cases where other pathologic findings were present in the fluid, especially an elevated colloidal gold curve. They were of the opinion that tryptophan, though it is not the sole factor, may be one of the agents responsible for the gold curve. In our series we obtained more positive tryptophan tests in syphilitic fluids with a paretic gold curve than without one. Though this relationship is not strictly parallel, we have found many more positive tryptophan tests in syphilitic fluid with a paretic gold curve than without one. This would explain its highest incidence in paresis. On the other hand, in cases with paretic gold curves but no syphilis, as in multiple sclerosis, the test has been negative. This convinces us that more than one factor is present in syphilitic spinal fluid to account for the colloidal gold curve and the tryptophan reaction. Perhaps the greater destruction of parenchymatous nerve tissue in paresis than in the other forms of syphilis results in a greater likelihood of the simultaneous production of the factors responsible for the tryptophan reaction and the colloidal gold curve.

Greenfield and Carmichael found negative results to the test even in cases with total protein up to 1 per cent. Baxter[2] reported negative tryptophan tests in fluids showing an increased cell count and positive Pandy. Brugi and Herbert[16] believed the results paralleled the increase in albumin content and obtained positive responses in fluids in which the albumin was greater than 30 mg. per 100 c.c. Steele and Nicole, and Duncan[9] found a close correlation between the globulin and the Boltz test, and in none of their cases was the result negative when the globulin was positive. Piotrowski's work disclosed no association between the globulin content and a positive Boltz reaction. Walker and Sleeper demonstrated a varying intensity of response with the amount of protein in the spinal fluid but stated that the relationship is not a direct one. In our series of the 18 cases in which the quantitative protein was 50 mg. per 100 c.c. or over, five with figures of 100, 50, 119, 50, and 110 mg. per 100 c.c., gave a negative tryptophan reaction. The remainder in this series were positive.

Kraus and Mezey included two malaria treated cases; one with paresis gave a negative test, the other with tabes gave a positive tryptophan test. Harris found in malaria-treated cases that, although the Wassermann reaction had in most instances become less strongly positive, the Boltz test showed no change. Steele and Nicole state that the Boltz test was altered in about half the malarial treated cases, if considerable alteration from the original paretic gold curve had occurred. Riebeling, on the other hand, thought that the results were more positive in persons with general paresis who were treated, than in those not treated. Three of the five with paresis, in our series, who had received malaria therapy six weeks to four years prior to the examination of the fluid, gave positive tryptophan reactions. We feel that our series of cases treated with malaria or chemotherapy is too small to warrant any definite conclusion in regard to the effect of the therapy on the tryptophan response.

Kraus and Mezey found a negative test in old standing neurosyphilis or in cases that had no signs of parenchymatous involvement. Schreus and Willms[26]

noted an increase in the reaction with the passing of time. They found the Boltz reaction positive in only 26 per cent of 37 cases of less than two years' duration, and in 56 per cent of 72 cases of more than two years' duration.

CONCLUSIONS

1. A total of 109, or 82 per cent, of 133 paretic fluids gave a positive tryptophan response. It was positive in 3 (100 per cent) cases of taboparesis, negative in 4 (100 per cent) cases of tabes, and positive in 5 of 13 or 46 per cent of cases of psychosis with meningovascular or cerebral syphilis.

2. The test is most often positive in cases of syphilis with a paretic gold curve, but does not occur in other diseases as multiple sclerosis where a paretic gold curve but no syphilis is found.

3. The test is not necessarily dependent upon a quantitative increase in the total protein of the spinal fluid.

4. In a control series of 102 cases in which there was no positive serology or history of syphilis, it was positive in only 2 cases, in one of cerebral arteriosclerosis, and in one of rheumatic fever.

5. From the literature we have tabulated the results of the Boltz and Riebeling tests which are probably dependent upon the tryptophan in the spinal fluid.

6. The spinal fluid must be clear because hemorrhagic, xanthochromic, or purulent fluids give false positive reactions.

We thank A. Wolfson, B.S., chemist at the Morrisania City Hospital, for aid in the examination of the spinal fluids.

REFERENCES

1. Aiello, G.: Sui prodotti di scissione dell' albumina nel liquido cefalo-rachidiano, Policlinico (sez. prat.) 29: 537, 1922.
2. Baxter, H.: Tryptophane Reaction as Aid to the Early Diagnosis of Meningeal Tuberculosis, Edinburgh M. J. 44: 663, 1937.
3. Blix, G., and Backlin, E.: On the Chemical Basis for Boltz Test on the Cerebro-spinal Fluid, Acta psychiat. et neurol. 2: 82, 1927.
4. Bock, H.: Über den Nachweis von Tryptophan in der Ruckenmarksflussigkeit bei Meningitis tuberculosa, Monatschr. f. Kinderh. 65: 41, 1936.
5. Bobeff, N. D.: Der diagnostische Wert der Tryptophan-Reaktion im Liquor cerebrospinalis bei Meningitis tuberculosa, Monatschr. f. Kinderh. 73: 358, 1938.
6. Boltz, O. H.: Studies on the Cerebro-spinal Fluid With an Acetic Anhydride-Sulphuric Acid Test, Am. J. Psychiat. 3: 111, 1923.
7. Brugi, A.: La reazione del triptofano nel liquor, Pensiero med. 12: 583, 1923.
8. Buchanan, J. A., and Ballweg, H.: The Tryptophane Reaction, New York State J. Med. 39: 58, 1939.
9. Duncan, A. G.: The Acetic Anhydride-Sulphuric Reaction for General Paresis, J. Ment. Sc. 73: 419, 1927.
10. Fildes, P., and Knight, B. C. J. G.: Tryptophane and the Growth of Bacteria, Brit. J. Exper. Path. 14: 343, 1933.
11. Greenfield, J. G., and Carmichael, E. A.: The Acetic Anhydride Test in Cerebrospinal Fluid, J. Neurol. & Psychopath. 7: 220, 1927.
12. Griep, W. A.: Tryptophan Reaction in Cerebrospinal Fluid, Nederl. tijdschr. v. geneesk. 81: 5610, 1937.
13. Grossman, S.: The Value of Simple Laboratory Tests in the Diagnosis of Neurosyphilis, J. Ment. Sc. 71: 439, 1925.
14. Guglielmin, G.: La reazione del triptofano nella meningite tubercolare, Tubercolosi 30: 127, 1938.
15. Harris, J. S.: A Simple Test of Diagnostic Value in General Paresis, Brit. M. J. 1: 136, 1926.
16. Herbert, F. K.: The Acetic Anhydride Test in Cerebrospinal Fluid, Brit. M. J. 2: 953, 1929.
17. Jequier, M., and Roten, J. L.: Recherche du Tryptophane dans le liquide cephalorachidien des meningites tuberculeuses, Schweiz. med. Wchnschr. 71: 95, 1941.

18. Kraus, M., and Mezey, K.: Tryptophanbestimmung im Liquor cerebrospinalis und deren Ergebnisse, Schweiz. Arch. f. Neurol. u. Psychiat. 42: 77, 1938.
19. Lichtenberg, H. H.: Tryptophane Test in Tuberculous Meningitis, Am. J. Dis. Child. 43: 32, 1932.
20. Massazza, A.: Sul contenuto nel "liquor" dei prodotti di scissione della molecola albuminoidea, Riv. di. pat. nerv. 32: 1, 1927.
21. Mouriz: Quoted by Kraus and Mezey.[18]
22. Piotrowski, G. L.: Acetic Anhydride Test of Spinal Fluid, Brit. M. J. 2: 457, 1929.
23. Pongratz, R.: Ueber die Bedeutung der Tryptophanprobe für die Diagnose der Meningitis tuberculosa, München. med. Wchnschr. 84: 814, 1937.
24. Riebeling, C.: Über den Nachweis von Tryptophanderivaten in Liquor, Deutsche Ztschr. f. Nervenh. 120: 64, 1931.
25. Rosenblatt, M. B.: The Tryptophane Test in Tuberculous Meningitis, Am. Rev. Tuberc. 29: 668, 1934.
26. Schreus, H. T., and Willms, E.: Über den Ausfall der Boltzreaktion in den verschiedenen Stadien der Lues, Dermat. Ztschr. 56: 8, 1929.
27. Schumacher, H. M.: Die Bedeutung der Tryptophanreaktion für die Diagnose der Meningitis tuberculosa, Ztschr. f. Kinderh. 56: 626, 1934.
28. Spillane, J.: The Tryptophane Reaction in the Spinal Fluid, Lancet 1: 560, 1937.
29. Steele, J. P., and Nicole, J. E.: The Reaction of the Cerebrospinal Fluid to the Acetic Anhydride Test, Brit. J. Ven. Dis. 2: 243, 1926.
30. Thomas, C.: The Boltz (Acetic-Anhydride) Test in Cerebro-Spinal Fluid, J. Ment. Sc. 76: 271, 1930.
31. Walker, B. S., and Sleeper, F. H.: Tryptophane Reactions in the Spinal Fluid, J. Lab. & Clin. Med. 12: 1048, 1927.

EFFECTS OF DESICCATION PROCEDURES ON THE CHEMICAL COMPOSITION OF FECES, URINE, AND MILK*

D. Maxwell Teague, M.S., Harry Galbraith, M.S., Frances Cope Hummel, M.S., Harold H. Williams, Ph.D., and Icie G. Macy, Ph.D.
Detroit, Mich.

THE art and science of dehydration have been practiced from the earliest times of mankind, for the sake of either concentration or preservation. In some types of chemical work it is desirable not only to concentrate and to preserve a given mixture or substance, but also to determine quantitatively the amount of water contained therein. The method of choice in the removal of water depends upon the completeness of desiccation desired and upon the chemical or physical alterations that may occur as the material is being dried.

Food and other biological products may be concentrated and preserved, or their water content may be studied by dehydrating with heat, at atmospheric or under reduced pressure. The loss in weight that takes place during the dehydration process, before constant weight is reached, is generally assumed to be due, primarily, to the vaporization of water. Drying at temperatures of 70° C. and upward to that of boiling water, however, may result in the volatilization of essential oils,[1] loss of nitrogen,[1-3] decomposition of fats and carbohyates[4-6] and, in some cases, the removal of water may be extremely time-

*From the Research Laboratory of the Children's Fund of Michigan, Detroit.
Some of the data included in this paper were presented before the Biological Division of the American Chemical Society, at the One-Hundredth Meeting of the Society, Detroit, Mich., September 9 to 13, 1940.
Received for publication, June 10, 1942.

consuming. After protein-containing substance has been dried to constant weight, the final product may still contain from 2 to 7 per cent water.[7, 8] The use of temperatures of 100° to 110° C. for dehydration may be accompanied not only by a loss of volatile substances but also by a gain in weight from oxidation of fats and carbohydrates.[1, 6]

Some of the difficulties in the removal of water may be obviated by drying at lower temperatures (50° to 70° C.) in an inert atmosphere of nitrogen or carbon dioxide under partial pressure, if special equipment is available for this purpose. If heating must be avoided, the classic method of drying at room temperature in a partial vacuum over a desiccant may be used—a method which is tedious, time-consuming, and impractical where large volumes of water are to be removed in a short time. For rapid determination of the moisture content of biological material, reflux distillation with xylene or toluene may be applied with a high degree of efficiency and accuracy.[9, 10] This latter method has no application in the preservation or concentration of foods for edible purposes, however, nor is it practical in connection with certain types of organic analyses.

In continuous metabolic balance experiments over many days, with several subjects, the number of specimens of foods, urine, and feces involved precludes chemical analysis of all samples immediately following collection. Indeed, under the usual circumstances of staff limitation, it is not practical even to attempt all the analyses concurrent with the collections. These conditions have constantly pointed out the need for procedures by which biological specimens may be preserved for future analysis in a state chemically equivalent to that existing at the time of collection. Increasing knowledge of physiologic processes and wider recognition of the great stability of biological substances in the dry state have emphasized the necessity for a more practical method of desiccation, for preservation with less danger of denaturation than the procedures previously mentioned.[11]

Although removal of water vapor from frozen specimens by means of a chemical desiccant was used at the beginning of the century, only recently has the cryochem process ("desiccation *in vacuo* from the frozen state by means of chemicals"[12]) been developed to a high degree of refinement. This process offers a rapid and safe procedure which permits the preservation of biological material in an undenatured form for long periods of time, provided the samples are properly stored. The purpose of this paper is to point out the advantages of the cryochem procedure in accumulating biological materials for future analysis, and to demonstrate how other methods of desiccation may alter the chemical composition of the same materials. The illustrations given portray the influence of different methods of dehydration upon distribution of free fatty acids and soaps in the feces and the total fat and nitrogen contents of feces, urine, and milk. The fecal fat excretion and its partition in the feces of normal children, determined on cryochem-desiccated feces, is reported in another paper.[13]

FECAL FAT

Fecal material is much more conveniently handled and accurately sampled for analyses in the dry state, when it can be pulverized and thoroughly mixed.

In determining the total lipid content of feces, whether the wet or dried stool material is used is of no consequence as far as the results are concerned, since most methods depend on primary saponification of the material. However, in the fecal fat partition it has been claimed[16, 17] that analyses on dried stools are inaccurate and that the lipid partition should be carried out only on the wet material. Tidwell and Holt[15] have shown, in a comparison of the fat partitions on wet and dry fecal samples, that there was no significant alteration in the fecal fat distribution when feces were dried at room temperature in air. In handling a large number of fecal samples over a period of time, it is impractical to attempt desiccation of them at room temperature in air. The usual procedure has been to dry feces under alcohol in a forced draught oven, maintaining a temperature below 70° C.

TABLE I

EFFECT OF DRYING METHOD UPON FAT COMPOSITION OF FECES

CONSTITUENT*	METHOD OF DRYING SAMPLE	MG. PER GM. DRIED FECES		CRITICAL RATIO	ANALYSIS OF VARIANCE	
		MEAN ± SE_M	SD ± SE_{SD}		F	F^1
Free fatty acid	Oven	42.86 ± 2.03	7.60 ± 1.44	13.61	190	9.07
	Cryochem	14.28 ± 0.55	2.06 ± 0.39			
Soap	Oven	54.64 ± 6.10	22.81 ± 4.31	3.05	61	9.07
	Cryochem	81.86 ± 6.50	24.34 ± 4.60			
Free fatty acid plus soap	Oven	97.50 ± 6.52	24.39 ± 4.61	0.14	0.19	9.07
	Cryochem	96.14 ± 6.69	25.05 ± 4.73			

*The fat constituents were determined on the two types of dried preparation from each of 14 samples.

Corresponding samples of the feces from a group of normal children* were oven and cryochem dried. Table I compares the results of free fatty acid and soap analyses of the two types of material. The average amounts of free fatty acids per gram of dried feces were 43 mg. for the oven-dried samples and 14 mg. for the cryochem-dried; the average soap contents were 55 and 82 mg. per gram, respectively. That real differences existed for both the free fatty acid and soap determinations between the values obtained upon the samples prepared in the two ways, is shown by the critical ratios and the Analysis of Variance[15] results. The conclusion apparent from the results is that during the process of drying in the oven, a portion of the soaps was hydrolyzed. This seems evident, also, when the total free fatty acid plus soap contents of the feces dried by the two methods are compared. The average total for the oven-dried material is 97 mg. per gram and for the cryochem-dried feces it is 96 mg. No real difference was found between the free fatty acid plus soap analyses made on samples prepared in the two ways, indicating that while the total remained unchanged there was a real change in its components. From these results it seems clear that when the fecal fat partition is to be determined, care must be exercised in selecting the method of desiccation if the analyses are to be performed on dried material.

*The experimental studies with normal children were made possible by the cooperation of the Methodist Children's Village, Detroit.

NITROGEN

One of the determinations most frequently performed on biological material is analysis for nitrogen, from the results of which the protein content may be calculated. During standing and dehydrating, the organic constituents are most susceptible to loss of nitrogen.[3] The effect of the cryochem procedure of desiccation on nitrogen content is illustrated by analyses of feces, urine, and milk.

Fecal Nitrogen.—The averages of results of determination of the nitrogen content of the feces of children who were subjects of a continuous metabolic balance study over a period of fifty-five days[14] are given in Table II. Each fecal sample represented a five-day period, and similar samples from each of several children were carefully collected and preserved, after preparing in the following manner: (1) A sulfuric acid digest[14] was made of one portion of the fresh feces and the nitrogen determined. This value has served as a standard for comparison. (2) A portion of the fresh feces was dried down, under alcohol, in a forced draught, steam heated oven below 70° C. (3) Another portion of the fresh feces was dehydrated by the cryochem process.

TABLE II

EFFECT OF DRYING METHOD ON NITROGEN CONTENTS OF FECES AND URINE

MATERIAL	METHOD OF PREPARING SAMPLE	NITROGEN*		CRITICAL RATIOS	
		MEAN ± SE_M	SD ± SE_{SD}		
Feces†	Acid digest	1380 ± 37	145 ± 26	Acid digest:cryochem dried	1.5
	Cryochem dried	1309 ± 31	120 ± 22	Acid digest:oven dried	5.3
	Oven dried	1125 ± 30	116 ± 21	Oven dried:cryochem dried	4.3
Urine‡	Fresh, untreated	9.52 ± 0.12	1.08 ± 0.08	Fresh:cryochem dried	0.5
	Cryochem dried	9.60 ± 0.11	1.05 ± 0.08		

*In milligrams per day for feces and milligrams per milliliter of fresh urine.
†From analyses of 15 samples.
‡From analyses of 88 samples.

From comparisons of the nitrogen contents of the preparations, drying in the oven caused a considerable loss of nitrogen, an average of 18 per cent of the nitrogen content of the acid digest of the fresh feces. The feces preserved by the cryochem procedure showed an average loss of only 5 per cent of the value determined for the acid digest, although the analyses were made two years after the samples had been dehydrated in the cryochem, and the loss was consistent for all samples. The children excreted in their feces, averages of 1,380, 1,309, and 1,125 mg. of nitrogen per day, as determined upon the acid digest, cryochem-dried, and oven-dried feces, respectively.

Statistical evaluation has been applied to the results obtained for the fecal nitrogen in 15 samples each of the acid digest, cryochem-dried, and oven-dried feces. The critical ratio (Table II) leaves some doubt as to the significance of the difference found between the nitrogen values for the acid digest and for the cryochem-dried samples, but the Analysis of Variance ($F = 51.68$; $F^1 = 8.86$) demonstrated a real difference. For the cryochem-prepared samples versus the oven-dried samples, the critical ratio (Table II) showed a real statistical difference, confirmed by the Analysis of Variance ($F = 89.67$; $F^1 = 8.86$). While the fresh samples contained slightly more nitrogen than the cryochem-

prepared samples, the latter had considerably greater nitrogen contents than the oven-dried samples. The real difference between the results obtained on the cryochem- and the oven-dried samples is evident from the values given in Table II and is confirmed by the critical ratio of 4.3.

Several explanations may be offered for the nitrogen loss shown by the cryochem-dried fecal material. Feces are known to contain approximately 40 per cent bacteria and volatile nitrogen compounds may already be present, or be formed even while the product is frozen. It is possible, also, that although the dried samples were stored in desiccators over desicchlora, they may have absorbed some moisture when the glass-stoppered bottles were opened to remove samples for other determinations. Despite the small loss of nitrogen from the feces dehydrated by the cryochem process and preserved for two years, it appears safe to conclude that this method of drying offers the best method for desiccating various biological materials with a minimum of chemical alteration.

Urinary Nitrogen.—A comparison has been made between the nitrogen contents of 88 samples of urine, as determined upon fresh and upon cryochem-dried samples. From the average data for these series of analyses the nitrogen contents of the two preparations appear similar (Table II). The averages were 9.52 and 9.60 mg. of nitrogen per milliliter of urine in the fresh and dried samples, respectively; the ranges of variation shown by the standard deviations (SD) correspond closely, and the critical ratio is only 0.5.

Milk Nitrogen.—Five samples of fresh milk were secured at weekly intervals and analyzed for nitrogen by the Kjeldahl method.[14] Portions of the same samples were dried by the cryochem procedure and the nitrogen similarly was determined. The averages for nitrogen, determined upon the dried and fresh samples, were 509 and 506 mg. per 100 Gm. of milk, respectively, which represents a difference of only 0.5 per cent in the nitrogen contents.

It should be pointed out in this connection that in determining the energy value of such substances as milk and urine in the bomb calorimeter, other methods of drying make it necessary to determine the nitrogen loss in drying and make a correction for this loss in calculating the heat of combustion of the original material. The cryochem desiccation process offers a means of eliminating the additional determination of nitrogen on the dried material and the nitrogen loss correction in the energy calculation.

SUMMARY

The removal of water from feces, urine, and milk by the cryochem process (dehydration *in vacuo* from the frozen state by means of chemicals) permits preservation of the dry material indefinitely in an undenatured form if stored under proper conditions. This method of dehydration has many advantages over oven-drying. Oven-drying at 70° C., and under, hydrolyzes the soaps in feces, causing exaggerated values for the free fatty acids and a reduction in the soaps, although the total free fatty acid plus soap values are the same for both methods of drying. The nitrogen contents of the cryochem samples of feces, urine, and milk approximate those of fresh specimens. In determining energy by the bomb calorimeter, the cryochem-dried material permits greater

accuracy in analyses and economy of time and materials, eliminating one nitrogen determination and correction for nitrogen loss in drying.

REFERENCES

1. Benedict, F. G., and Manning, Charlotte R.: The Determination of Water in Foods and Physiological Preparations, Am. J. Physiol. 13: 309, 1905.
2. Benedict, F. G., and Manning, Charlotte R.: The Determination of Water in Proteins, Am. J. Physiol. 18: 213, 1907.
3. Howe, P. E., Rutherford, T. A., and Hawk, P. B.: On the Preservation of Feces, J. Am. Chem. Soc. 32: 1683, 1910.
4. Pennington, Mary E., and Hepburn, J. S.: Studies on Chicken Fat. I. The Occurrence and Permanence of Lipase in the Fat of Common Fowl (Gallus domesticus), J. Am. Chem. Soc. 34: 210, 1912.
5. Smith, C. A., Miller, R. J., and Hawk, P. B.: Changes in the Fat Content of Feces Preserved by Freezing Without the Addition of a Preservative, J. Biol. Chem. 21: 395, 1915.
6. Nelson, O. A., and Hulett, G. A.: The Moisture Content of Cereals, J. Ind. Eng. Chem. 12: 40, 1920.
7. Gortner, R. A.: The State of Water in Colloidal and Living Systems, Faraday Soc. Trans. 26: 678, 1930.
8. Rimington, C.: Discussion re the State of Water in Colloidal and Living Systems, Faraday Soc. Trans. 26: 699, 1930.
9. Dean, E. W., and Stark, D. D.: A Convenient Method for the Determination of Water in Petroleum and Other Organic Emulsions, J. Ind. Eng. Chem. 12: 486, 1920.
10. Miller, A. T., Jr.: Studies on Tissue Water. I. The Determination of Blood Water by the Distillation Method, J. Biol. Chem. 143: 65, 1942.
11. Flosdorf, Earl W., and Webster, G. W.: The Determination of Residual Moisture in Dry Biological Substances, J. Biol. Chem. 121: 353, 1937.
12. Flosdorf, Earl W., and Mudd, Stuart: An Improved Procedure and Apparatus for Preservation of Sera, Microörganisms and Other Substances—The Cryochem Process, J. Immunol. 34: 469, 1938.
13. Williams, Harold H., Endicott, Elizabeth N., Shepherd, Marion L., Galbraith, Harry, and Macy, Icie G.: Fat Excretion by Normal Children and Possible Relationships With Calcium Retention, J. Nutrition. In press.
14. Macy, Icie G.: Nutrition and Chemical Growth in Childhood. Vol. I. Evaluation, Springfield, Ill., 1942, Charles C Thomas.
15. Snedecor, George W.: Calculation and Interpretation of Analysis of Variance and Covariance, Ames, Iowa, 1934, Collegiate Press.
16. Fowweather, F. S.: The Determination of the Amount and the Composition of the Fat of Feces. I. Investigation of a Wet Method and Comparison With the "Dry" Method, Brit. J. Exper. Path. 7: 7, 1926.
17. Fowweather, F. S.: The Determination of the Amount and the Composition of the Fat of Feces. II. The Composition of the Fat of the Feces of the Normal Adult as Ascertained by the "Wet" Method Together With Some Results in Certain Pathological Conditions, Brit. J. Exper. Path. 7: 15, 1926.
18. Tidwell, Herbert C., and Holt, L. Emmett, Jr.: The Estimation of the Total Lipids and the Lipid Partition in Feces, J. Biol. Chem. 112: 605, 1936.

ON THE PURIFICATION OF GUM GHATTI*

L. F. Wicks, M.S., St. Louis, Mo.

ALTHOUGH the use of Nessler's reagent dates back to 1856, it was apparently Chiles[1] in 1928, who first emphasized the great gain in stability of nesslerized solutions when gum arabic was present. By its inclusion he successfully nesslerized Kjeldahl digests containing concentrations of ammonium ion far greater than is otherwise possible. Such application of a suitable protective colloid was indicated, of course, once the Nessler reaction color was suspected of being a colloidal suspensoid. Prussian blue sols, present in a micro glucose method, were stabilized with gum ghatti by Folin[2] in 1929. Looney[3] in 1930 employed this same gum for his simple blood urea method by direct nesslerization. A year later Walters[4] applied it to other nesslerized solutions, claiming gum ghatti to be much superior to Chiles' gum arabic, of which it is a variety.

As would be expected, the natural gums are very variable substances, and most samples contain interfering compounds, which can cause reduction of the double iodide of Nessler's reagent with the separation of what seems to be mercurous compounds. When Nessler's reagent is added to a water solution of most natural gums, a greenish-yellow turbidity appears, and slow precipitation follows. Fortunately, the colored ammonia complex of the Nessler reaction is less easily reduced but still turbidity often develops. These reducing materials are doubtless responsible for the limited use of the gums as an aid to nesslerization and for disagreement as to its value. For example, Looney found gum ghatti to retard greatly the rapid turbidity that follows in almost all direct nesslerization urea methods, while Hawk and Andes[5] reported increased turbidity.

Several investigators have tried to remove the objectionable reducing substances in the gum solution with only partial success. Oxidation was an evident approach. Chiles mentioned using sodium peroxide but discarded it and simply mixed a portion of Nessler's reagent with a greater volume of crude gum solution and waited for it to clear. Doneen[6] suggested Chiles' method of preparation whenever the gum solution proved too crude. Folin,[2] in preparing gum ghatti solution for the iron-reduction glucose method cited above, oxidized with potassium permanganate. Other analysts, such as Walters, and Lieboff and Koppel,[7] have precipitated the gum from aqueous solution by strong alcohol. Most workers, however, including Looney, and Daly,[8] used, if any, a crude solution. Herzfeld,[9] and Nichols and Willits[10] employed gelatin which has a much lower gold number[11] than the gums. We have tried tragacanthin, the soluble portion of gum tragacanth,[12] without much satisfaction.

*From the Research Department of the Barnard Free Skin and Cancer Hospital, St. Louis.

Received for publication, June 15, 1942.

EXPERIMENTAL

Although most samples of "soluble gum ghatti," or of gum arabic, include contaminations like woody fibers, earthy material, insoluble gum, and coloring matter, the impurities most objectionable from the view point of nesslerization are calcium, magnesium, and ammonium compounds, but especially the reducing substances.

The first problem is that of dissolving the gum. Some texts give directions for suspending the sample at the top of a cylinder of cold water where it requires days to disperse. Others lengthily stir the powdered gum with cold water, a method by which solutions up to 10 per cent are prepared, an unnecessary concentration, which is as viscous as syrup and cannot possibly be filtered or centrifuged. It is not only easier but better to prepare the solution hot, for it is thus somewhat less frothy and a little lower in reducing substances. Snell and Snell[13] recognized this, for while their directions call for a laborious dissolving of the gum in cold water, they then recommend heating the solution over a boiling brine bath "to destroy reducing enzymes."

We also attempted the destruction of the reducing substances by oxidizers, such as potassium mercuric iodide, triiodide, permanganate, hydrogen peroxide, etc., without much satisfaction. Permanganate, as used earlier by Folin, gives only partial improvement, and the deep brown oxide of manganese remains peptized in the gum solution.

The expedient of mixing some Nessler's reagent with a much greater volume of gum solution to clear out the reducing substances was found to have certain disadvantages. The alkali of the reagent seems at first to increase the reducing power of the gum solution, doubtless by hydrolysis and other reactions. Consequently, it is not practical to incorporate a small amount of protective gum solution with undiluted Nessler's reagent stock before use. Upon mixing the solutions, a very pronounced turbidity develops. After many days, the supernatant liquid is clear above a greenish or black residue of mercurous iodide or mercury. However, despite decomposition of the added Nessler's reagent and part of the gum, the final solution, though alkaline, is substantially lower in reducing substances. Unfortunately, use of such gum ghatti solution requires a special Nessler's reagent containing less alkali and more of the double iodide.

The alcohol precipitation technique is evidently based upon the assumption that the chief interfering substances are the salts associated with the gums. Addition of alcohol to an aqueous solution of a gum up to a concentration of 60 per cent or more[11] causes separation. Long contact of the precipitated gum with strong alcohol lowers its solubility in water. The sticky, demineralized mass is then dried, pulverized, and dissolved in water. This is a messy procedure and one which removes the salts but only a small portion of the reducing substances. One can prepare a better (but not good) gum ghatti solution by simply washing the powdered crude sample with hot alcohol, drying, dissolving in water to a 1 per cent solution, treating with permutit, and filtering.

True gums can also be precipitated from aqueous solution by basic lead acetate, but they are thereby denatured and are no longer water-soluble.

Turning then to the possibility of removing the interfering materials by adsorption, a variety of adsorbing agents was tried. Alumina gave fair results,

but this oxide is indicated[14] to attach pentosan gums. The clays, kaolin, and Lloyd's reagent were slightly effective, but they were so finely divided that it was almost impossible to separate the suspended particles. Silica in the form of kieselguhr had the same fault; ground silica gel did not, of course, but neither was it very active. The zeolite, permutit, besides removing calcium, magnesium, and ammonia, seemed to adsorb some coloring matter and reducing substances. However, adsorbtive carbons proved most satisfactory, especially purified bone charcoal, which we found can take out the greater portion of the reducing substances.

SUGGESTED PROCEDURE

Bring the desired volume of distilled water to a boil, and then transfer the beaker to a steam bath. Add enough of a good grade of powdered gum ghatti (e.g., Coleman and Bell "gum ghatti, soluble") to make a 1 per cent solution, and heat with frequent stirring for about one-half to three-quarters hour, or until all soluble material appears to be dissolved. (Heating over a direct flame can char the gum.) While still hot, filter with suction through a couple thickness of closely-woven cloth pressed down snugly in a Büchner funnel with a disk of wire screen. (Only the grosser particles will be retained, but even the coarsest crepe filter paper would soon clog.) Return the gum solution to the steam bath, add about 6 to 8 Gm. of dry, acid-purified bone charcoal powder per 100 c.c., cover with a watch glass, and heat with occasional stirring for one-half to one hour. Oddly, after the charcoal treatment, the viscosity of the gum solution is apparently greatly reduced, as it is then much more readily filtrable (although Rakuzin[14] states that gum arabic at least is not adsorbed by charcoal). Filter while hot through a fast crepe paper (e.g., Reeve Angel 202) in the Büchner funnel. The filtrate may be grayish, due to fine particles of carbon. If so, change the filter paper to a retentive one (e.g., Munktell OA) and refilter. For each 100 c.c. of the cool filtrate, add about 3 to 4 Gm. of *good* permutit (e.g., "according to Folin"—or purify as described by Hawk and Bergeim[15]). Agitate gently for around ten minutes and filter with crepe paper and suction.

"Bone charcoal" as often sold may consist of a great deal of calcium compounds (including sulfides) and a little partly decomposed organic matter along with the porous carbon. Use of such material can render the gum solution more impure than before. If acid-treated bone charcoal is not available, an ordinary grade (e.g., Mallinckrodt No. 4396) must be purified, as described by Hawk and Bergeim.[15] Over 80 per cent of the "charcoal" may dissolve in the hot, dilute hydrochloric acid. To avoid prolonged washing, the residue can be removed from the Büchner funnel after a couple hot water rinsings, suspended in a little sodium bicarbonate solution (test with litmus), then refiltered and washed again in the funnel. It is naturally important that the charcoal be free of any strong acid. The final product may be used over if washed well with hot water and dried in the oven. The permutit may also be washed, repurified, and used again.

ALTERNATIVE PROCEDURE

A method not quite as effective as the foregoing, but much more convenient, is as follows: Prepare the gum solution on the steam bath as outlined

but to only 0.5 per cent concentration. Filter hot through cloth as described previously, and add about 2 to 3 Gm. of a good alkali-free "activated" wood charcoal powder (e.g., "Activated Charcoal, Merck") per 100 c.c. Heat on the steam bath with frequent stirring for at least one-half hour. Filter hot with suction through crepe filter paper and then through a more retentive paper to catch the finer carbon particles. With wood charcoal these particles are smaller and lighter, and hence more difficult to remove. A lower concentration of the gum solution is necessary. The permutit treatment may often be omitted when wood charcoal is the adsorbing agent.

All the experiments outlined here were made by nesslerizing in the old Folin-Wu tubes in the presence of 1 c.c. of 1:1 sulfuric acid, and with the Koch-McMeekin reagent as prepared with mercuric oxide,[16] itself a rather stable reagent. As little as 5 c.c. of the purified 0.5 per cent gum ghatti will stabilize 1.5 mg. of nitrogen per 50 c.c. for hours, and 10 c.c. (or equivalent in 1 per cent) will serve for as high as 2.5 mg.

In adding the gum solution to the micro-Kjeldahl digest to be nesslerized, it is well to dilute that acid solution first to avoid any chance of possible hydrolysis of the pentosan gum.

Gum solutions will slightly lower the intensity of the Nessler color, doubtless due to the influence on particle size. They must naturally be added to the standard also.

As some molds grow readily in gum solutions, it is best to include a preservative. This must be one which causes no reduction when mixed with Nessler's reagent. Chloroform definitely gives marked reduction from contact with the alkali (formate?). Walters used water saturated with thymol, a good preservative but one which can cause a slight reduction. Toluol was found most satisfactory, only a few drops being required to prevent mold growth without refrigeration. (It is possible, by adding too much toluol and shaking very vigorously, to form a toluol emulsion.)

SUMMARY

Reducing substances naturally present in most samples of gum ghatti will often cause turbidity of Nessler's reagent when that protective colloid is employed. The greater part of the interfering materials can be removed by treatment of the hot gum solution with acid-washed bone charcoal (or less well by wood charcoal) followed by permutit. These procedures are described.

REFERENCES

1. Chiles, H. M.: Direct Nesslerization of Kjeldahl Digestions, J. Am. Chem. Soc. 50: 217, 1928.
2. Folin, O.: Supplementary Note on the New Ferricyanide Method for Blood Sugar, J. Biol. Chem. 81: 231, 1929.
3. Looney, J. M.: The Determination of Blood Urea by Direct Nesslerization, J. Biol. Chem. 88: 189, 1930.
4. Walters, L. C.: The Colorimetric Estimation of Nitrogen by Direct Nesslerization, Australian J. Exper. Biol. & M. Sc. 7: 113, 1930.
5. Hawk, F. L., and Andes, J. E.: A Simplified Method for the Determination of Blood Urea, Am. J. Clin. Path., Tech. Supp. 8: 153, 1938.
6. Doneen, L. D.: A Micro Method for Nitrogen in Plant Material, Brit. Chem. Abstr. A.: 327, 1933.
7. Lieboff, S. L., and Koppel, D.: A Study of the New Folin Micromethod for the Determination of Blood Sugar, J. Lab. & Clin. Med. 15: 151, 1929.

8. Daly, C. A.: The Determination of Non-Protein Nitrogen, J. Lab. & Clin. Med. 18: 1279, 1933.
9. Herzfeld, E.: Über Eine Einfache Modifikation der Nessler-Kolorimetrie, Mikrochem. 17: 155, 1935.
10. Nichols, M. L., and Willits, C. O.: Reactions of Nessler's Solution, J. Am. Chem. Soc. 56: 769, 1934.
11. Thomas, A. W.: Colloid Chemistry, New York, 1934, McGraw-Hill Book Co.
12. Norman, A. G.: Studies on the Gums. II. Tragacanthin, the Soluble Constituent of Gum Tragacanth, Biochem. J. 25: 200, 1931.
13. Snell, F. D., and Snell, C. T.: Colorimetric Methods of Analysis, New York, 1937, D. Van Nostrand Co., Vol. II.
14. Rakuzin, M. A.: The Adsorption of Organic and Inorganic Colloidal Electrolytes, Chem. Abstr. 17: 3635, 1923.
15. Hawk, P. B., and Bergeim, O.: Practical Physiological Chemistry, ed. 11, Philadelphia, 1937, P. Blakiston's Son & Co.
16. Wicks, L. F.: A Cheaper Nessler's Reagent by the Use of Mercuric Oxide, J. Lab. & Clin. Med. 27: 118, 1941.

A NEW MODIFICATION OF THE THUNBERG METHOD FOR THE DETERMINATION OF DEHYDROGENASES IN TISSUES*

Ulrich Friedemann, M.D., and Alvin Hollander, M.S., Brooklyn, N. Y.

FOR the past two years we have carried out systematic studies on the effect of toxins and viruses on the dehydrogenase systems of tissues. The results of these investigations will be published in a separate paper. The classical method for this purpose, that described by Thunberg, requires special tubes which must be thoroughly evacuated and shaken during the entire course of the experiment. It was soon felt that this was too cumbersome for large-scale experiments. A modification was designed, therefore, which, without apparent disadvantages, permits the performance of many tests in a relatively short period of time. Instead of using evacuated tubes, the reagents were mixed with melted agar and the reaction was observed in the solidified medium. By this device anaerobic conditions were obtained, and, at the same time, sedimentation of the tissues was prevented.† Since we feel that this method may have other applications, we are presenting the technique in some detail.

Reagents.—

I. 2 per cent agar containing 0.5 per cent dibasic sodium phosphate and adjusted to pH 7.4.

II. 0.002 M methylene blue in distilled water.

III. Solutions of the various substrates. Succinate was used in 5 per cent solution and the others, citrate, glycerophosphate, glucose, and lactate, in equimolar concentrations. The pH of all solutions was adjusted to 7.1 with sodium hydroxide.

---

*From the Division of Bacteriology, the Jewish Hospital of Brooklyn.

Received for publication, June 15, 1942.

†After the elaboration of this method, Isaacs and Nussbaum[1] reported another modication of the Thunberg method for the determination of the dehydrogenases of bacteria. They suspended resting bacteria in saline and excluded oxygen by overlayering the fluid with vaseline. In our earlier experiments we used mineral oil for the same purpose but did not find it suitable for tissues. Because of the constant shaking required to prevent sedimentation of the tissue elements, the continuity of the oil layer was disrupted and oxygen gained access to the underlying fluid. The use of agar, therefore, is an essential feature of our technique.

IV. Tissue suspensions. The organs were finely ground with sand and 10 per cent suspensions were made in saline.

PROCEDURE

The agar was melted and 2.5 c.c. were put into each tube. When the agar had cooled to 42° C., 0.5 c.c. of methylene blue and 1 c.c. of substrate (both warmed to 37° C.) were added. This mixture was then added to 1.25 c.c. of tissue suspension, also previously warmed to 37° C. Complete admixture of the components were accomplished by rather vigorous rotation between the palms. Immediately afterwards the tubes were plunged into a freezing mixture of ice and salt in water and, when the agar had solidified, kept for an additional ten minutes in a refrigerator.

The tubes were then put into a water bath at 38° C. and observed for the decolorization of the methylene blue. If the tissues were ground finely enough, the time at which the end point of the reaction was reached could be determined accurately. Owing to some slight diffusion of oxygen from the air into the agar a narrow, well-demarcated zone of blue coloration was present at the surface but did not interfere with the reading of the results. In order to determine the degree of anaerobiasis in the agar, 1.25 c.c. of tissue suspension and 1 c.c. of a 1 per cent solution of paraphenylenediamine hydrochloride (neutral to litmus) were added to 2.5 c.c. of agar and handled as described above. A hazy gray color appeared in the agar indicating the presence of a trace of oxygen. This color, however, disappeared within a few seconds except for a well-defined, narrow black band at the surface. We may conclude from this observation that within a few seconds after the start of the experiment, the agar is free from oxygen. The quantitative relationship between the time required for decolorization and the amount of dehydrogenase will be discussed in a subsequent paper.

In order to test the reproducibility and consistency of the results, tests were done with the organs of two animals using succinate as a substrate. In both series each individual organ was tested five times under exactly the same experimental conditions. From the experimental data the arithmetic means and the standard deviations were calculated and are recorded in Table I.

TABLE I

DETERMINATION OF SUCCINASE ACTIVITY IN VARIOUS SAMPLES OF THE SAME ORGAN

ORGAN	EXPERIMENT I		EXPERIMENT II	
	MEAN*	STANDARD DEVIATION*	MEAN*	STANDARD DEVIATION*
Kidney	19.8	± 0.68	9.1	± 0.26
Liver	18.8	± 0.68	10.4	± 0.20
Heart	16.0	± 0	10.9	± 0.90
Brain	28.4	± 3.4	30.2	± 1.99

*Time in minutes.

The values of the standard deviations show that the results in each experiment with kidney, liver, and heart are consistent. In the experiments with brain, the results vary more considerably, although the experimental conditions were kept strictly constant. This probably is due to the well-known chemical instability of brain suspensions.

It may be seen from these two experiments that there are large differences in the dehydrogenase contents of the organs of various animals. Table II shows the results of experiments with the organs of 20 animals. Again we confine ourselves to determinations of succinase.

TABLE II

DETERMINATION OF SUCCINASE ACTIVITY IN THE ORGANS OF 20 ANIMALS

ORGAN	MEAN*	STANDARD DEVIATION*
Kidney	19.2	± 4.13
Liver	21.7	± 5.53
Heart	24.9	± 6.08
Brain	46.8	± 15.47
Adrenals	36.8	± 8.72

*Time in minutes.

These results show that any comparison between normal and pathologic organs can be made only on a statistical basis.

SUMMARY

A new modification of the Thunberg method for the determination of dehydrogenases in tissues has been reported. Tissue suspensions, methylene blue, and substrates are mixed with melted agar and decolorization observed in the solidified medium. The agar provides anaerobic conditions and prevents sedimentation of the tissue elements. The simplicity of this method permits the carrying out of large-scale experiments in a short period of time.

REFERENCE

1. Isaacs, M. L., and Nussbaum, M.: J. Bact. 41: 24, 1941.

A PHOTOELECTRIC METHOD FOR THE DETERMINATION OF POTASSIUM IN BLOOD SERUM*

JOSEPH M. LOONEY, M.D., AND CORA G. DYER, M.S., M.T., WORCESTER, MASS.

THE growing importance of potassium in physiologic investigations is attested to by the multiplicity of methods proposed for its determination. This fact is also evidence that none of the methods so far used has received general acceptance as wholly satisfactory and reliable. In our studies on the metabolism of schizophrenic patients we have used many methods, but most of our results have been obtained by the silver cobaltinitrite method of Breh and Gaebler,[1] modified by the procedure of Taylor,[2] for handling the standard. We have not been entirely satisfied with this method as the degree of error was too high to allow the detection of small but significant changes. When the extinction coefficient for the color developed by this method on known potassium solutions was determined on different days, there was a marked divergence of values, especially at the higher levels. The results for different concentra-

*From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester.

Received for publication, June 22, 1942.

tions of potassium obtained at the same time followed Beer's law but were not consistent from day to day. Truzkowski and Zwemer[3] subjected the method to a critical analysis and recommended that the precipitation be carried out between 17° and 22° C. Robinson and Putnam,[4] however, claimed that greater sensitivity can be obtained by precipitating from a 30 volume per cent alcohol medium at 4° to 6° C.

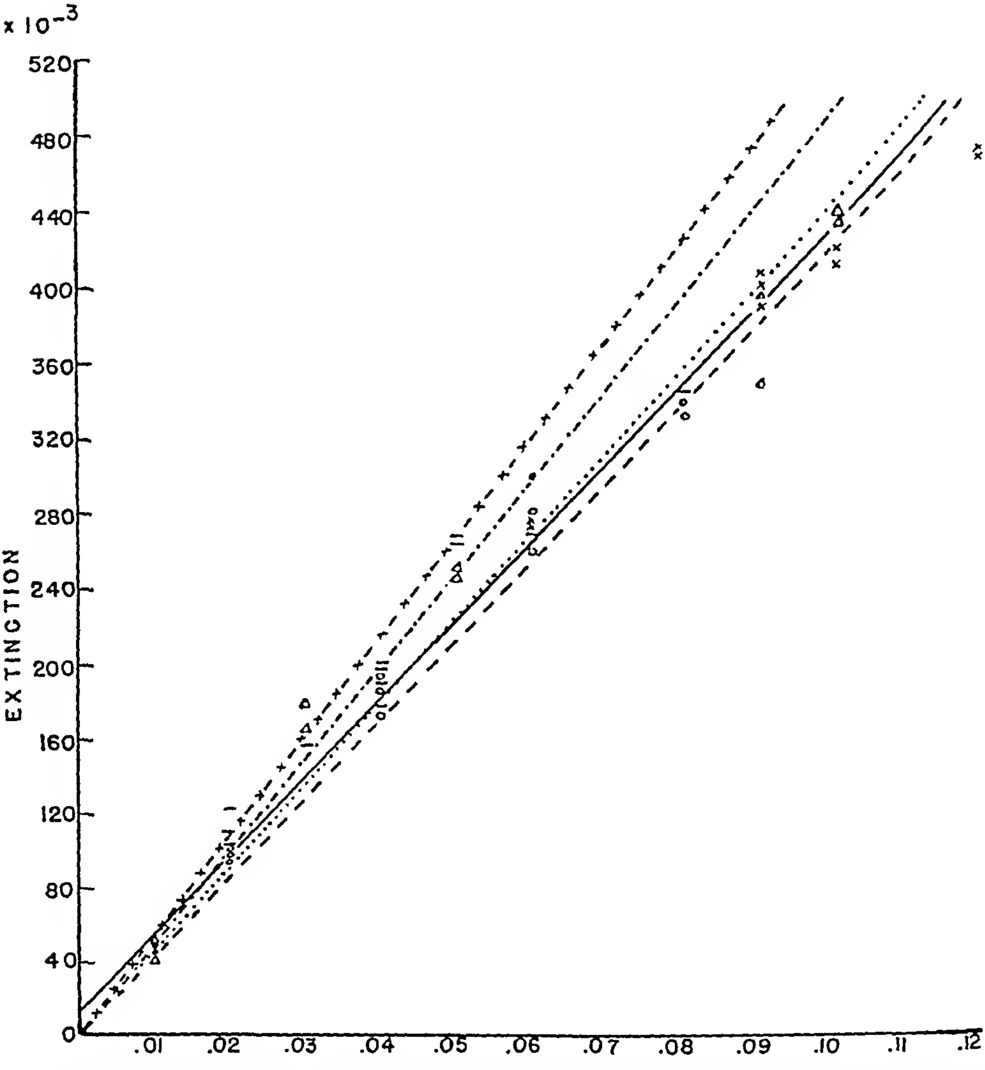


Fig. 1.—Variations in extinction produced by same amounts of potassium on different days with sulfanilic acid and α-naphthylamine. Temperature of precipitate held 20° to 23°. 1 c.c. of $AgNO_3$ = 20 c.c. of cobaltinitrite.

Harris[5] used a modification of the Breh and Gaebler method by precipitation from a 14 per cent alcoholic solution in a water bath at 20° C. for one-half hour. This period is much shorter than that recommended by most authors, and we have found it insufficient to obtain complete precipitation in very dilute solutions. Harris stated that the temperature could vary from 16° to 25° C. without change in the potassium/nitrite ratio. Our results indicate that the optimum range is between 18° and 20° C., since the potassium/nitrite ratio is

not constant above 23° C. and there is a tendency for fine needles of silver nitrite to precipitate below 16°. We have adopted his procedure of washing with an alcohol-ether-water mixture, since it is definitely superior in preventing flotation of the fine precipitate.

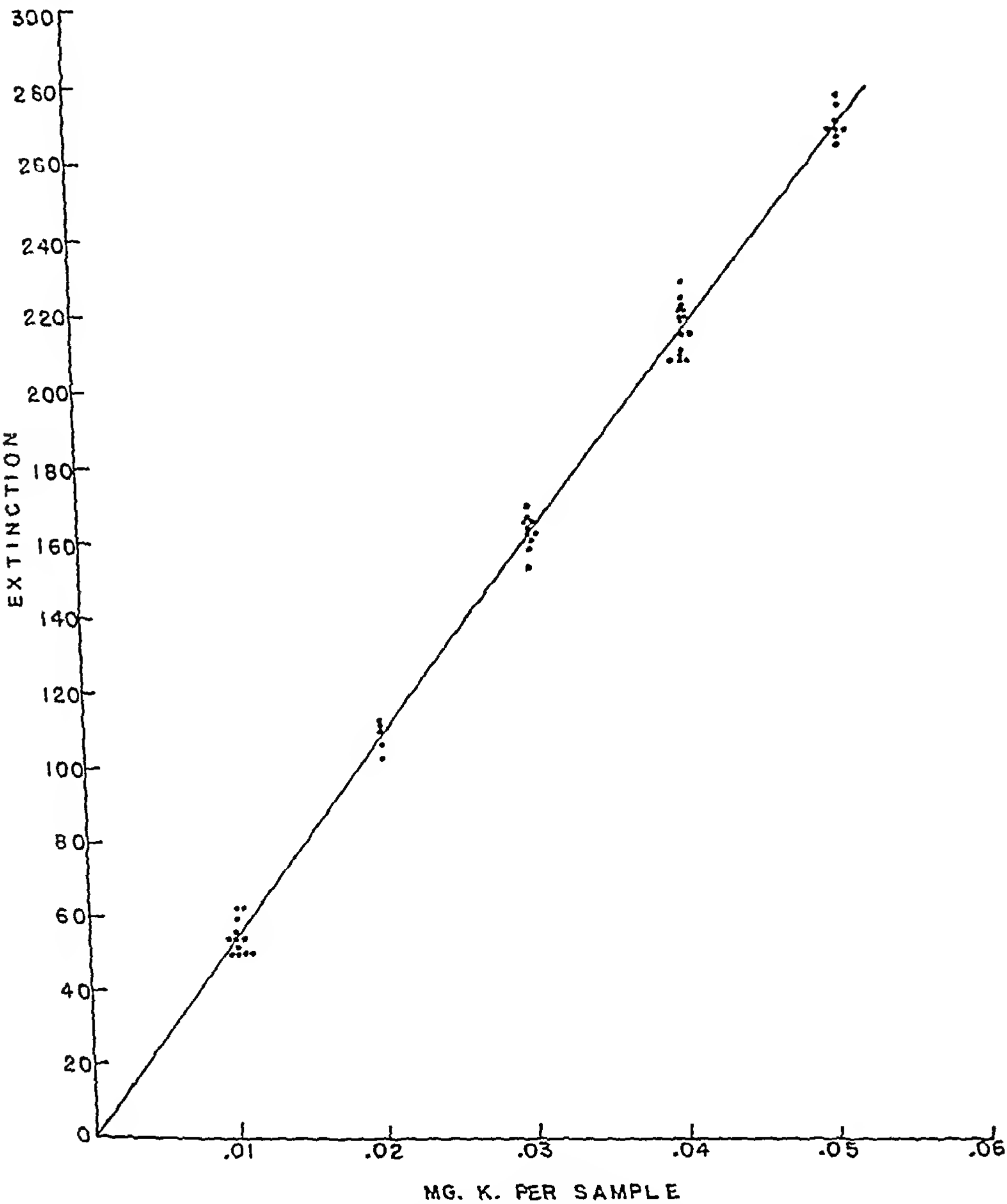


Fig. 2.—Extinction values for potassium silver cobaltinitrite with sulfanilamide and N-1 naphthylethylenediamine dihydrochloride.

The preparation of protein-free precipitates was also investigated. The commonly used Folin-Wu tungstic acid procedure plus silver nitrate was frequently followed by slightly cloudy filtrates which gave inconsistent results. The use of zinc sulfate and sodium hydroxide in combination with silver nitrate gave clear filtrates, but the results on known solutions were usually too high.

The use of sulfosalicylic acid plus silver nitrate was ruled out, because the results were high due to a reaction between the acid and the coupling agents used for the subsequent colorimetric procedure. Trichloroacetic acid could not be used, since all the samples that we tried gave turbid solutions with silver nitrate.

Weichselbaum, Somogyi, and Rusk[6] recommended the use of copper sulfate to replace the sulfuric acid in the preparation of filtrates. Our experience indicates that this method gives satisfactory results. They also recommended changes in the preparation of the silver cobaltinitrite reagent, but we were not able to obtain the results that they claimed for this modification and, therefore, returned to the use of the reagent of Breh and Gaebler.[1]

The chief difficulty in the use of a photoelectric colorimeter and a *standard* absorption curve arises from the variability in the amount of color produced by identical amounts of potassium on different days. This variability, as is shown in Fig. 1, arises from changes which occur in the solutions of sulfanilic acid and α-naphthylamine. Another disadvantage of these reagents is that the color produced is not stable and begins to fade after it attains its maximum development in about five to ten minutes.

It was felt that the substitution of the coupling reagent of Marshall[7] N-1 naphthylethylenediamine dihydrochloride for α-naphthylamine and sulfanilamide for sulfanilic acid might be advantageous. Shinn[8] has found these substitutions to give superior results in the determination of nitrite in foods, water, and sewage. We have been able to confirm her findings with regard to the permanence of the color produced by the Marshall reagents. Whereas the sulfanilic acid and α-naphthylamine showed a fading from an absorption reading of 77.2 per cent to 70.8 per cent on standing for twelve hours, the same amount of nitrite with the Marshall reagents showed a decrease in color only from 82.5 per cent to 81.8 per cent. The intensity of color was also considerably greater with the latter reagents. Although Shinn stated that the reagents would give satisfactory results for one month, we have found that values cannot be relied upon after a week when using the standard curve, and so we prepare these solutions fresh each week. If two standard solutions are run through at the same time as the unknown solutions, then satisfactory values can be computed from the line drawn through these two points for solutions kept up to a month. It should be noted, however, that the line must pass through the origin, and if this does not occur, some error has been made and new standards must be run.

TABLE I

Mg. of K per 100 ml. final volume	0.002	0.004	0.006	0.008	0.010	0.012	0.016	0.018	0.020	0.024
No. of samples	13	5	11	14	8	7	6	5	4	3
Mean extinction	0.054	0.108	0.162	0.216	0.270	0.276	0.338	0.399	0.428	0.471
K	22.5	22.5	22.5	22.5	22.5	19.0	17.5	18.0	17.5	16.0

PROCEDURE

All glassware must be kept scrupulously clean. We have found it convenient to keep a set of glassware for use in potassium determinations only. The presence of ammonia will give falsely high results. Therefore, ammonia fumes must be avoided and tubes, must be capped at all times when not in manipulation. The silver concentration is also important, since an excess of

silver as well as low temperatures during the cobaltinitrite precipitation will give long needlelike crystals of silver nitrite which interfere with the results. For this reason, we have reduced the silver content of Breh and Gaebler's reagent by one-half.

A. *Precipitation of Protein and Chloride.*

1. To a clean test tube, add
   0.5 c.c. of serum,
   7.0 c.c. of distilled water,
   1.0 c.c. of 1.5% sodium tungstate, and mix
   1.0 c.c. of 2.5% copper sulfate.
   Stopper and shake well. Then add
   0.5 c.c. of 2.5% silver nitrate.
   Restopper and shake again.
2. Let stand for fifteen to twenty minutes and filter through a Whatman No. 5 paper. The first portion of the filtrate should be refiltered through the same paper in order to be sure of an absolutely clear solution, since a cloudy filtrate apparently gives a low result.

B. *Precipitation of Potassium With Silver Cobaltinitrite.*

1. To a clean 15 c.c. graduated centrifuge tube, add
   3 c.c. of the protein chloride-free filtrate,
   1 c.c. of 95% alcohol, and
   1 c.c. of distilled water.
2. Place in a water bath at 18° to 22° C. for five minutes.
3. Add 2 c.c. of silver cobaltinitrite reagent, cap and mix (by tapping, not by inversion), and replace in the water bath for two hours.
4. Centrifuge for fifteen minutes at 2,800 r.p.m.

C. *Washing the Precipitate.*

1. With a capillary pipette remove the supernatant fluid to the 0.2 c.c. mark.
2. Wash with 7 c.c. of wash reagent, rinsing down the sides and disturbing the precipitate as little as possible. If the tube is slanted, the remaining 0.2 c.c. of fluid will mix with the wash solution.
3. Centrifuge for fifteen minutes, decant, invert, and drain over filter paper for five minutes. Wipe the mouth of the tube.
4. Repeat this washing and draining twice more.

(After this stage, and only at this point, the procedure may be interrupted and the tubes allowed to stand overnight if one drop of distilled water is added to each tube to prevent the precipitate from drying up.)

D. *Digestion.*

1. Add 10 c.c. of approximately 0.2 N sodium hydroxide, breaking up the precipitate if possible.
2. Heat in a boiling water bath for ten minutes. A black precipitate remains.
3. Cool, make up to 10 c.c. with water, stopper, and mix. Replace stopper with centrifuge cap and centrifuge for five to ten minutes.

TABLE II

EXP. NO. AMOUNT SERUM USED	SERUM K (MG./100) VALUES	SERUM K (MG./100) AVG.	MG. OF K ADDED 100 ML.	TOTAL K OBTAINED VALUES	TOTAL K OBTAINED AVG.	TOTAL CALCU-LATED	ERROR OF TOTAL (%)
1 ½ c.c. serum	20.78 20.46 20.56	20.60	4.0	23.56 23.56	23.56	24.60	-4.2
			8.0	28.18 28.18	28.18	28.60	-1.5
			12.0	30.88 31.12	31.00	32.60	-4.6
2 ½ c.c. serum	20.46 20.66	20.56	4.0	24.66 24.12	24.39	24.56	-0.69
			8.0	28.66 28.18	28.42	28.56	-0.49
			12.0	32.22 32.22	32.22	32.56	-1.0
3 ½ c.c. serum	22.00 22.56	22.28	4.0	25.12 26.00	25.52	26.56	-2.8
			8.0	29.78 29.56	29.67	30.28	-2.0
			12.0	32.78 32.78	32.78	34.28	-4.3
4* ½ c.c. serum	20.18	20.18	4.0	24.12 24.12	24.12	24.18	-0.24
			8.0	27.22 27.78	27.50	28.18	-2.4
			12.0	31.12	31.12	32.18	-3.3
5† ½ c.c. serum	21.44 19.66 20.00	20.36	4.0	25.00 24.56	24.78	24.36	+1.7
			8.0	28.18 28.34 28.34	28.29	28.36	-0.25
			12.0	32.00 32.12	32.06	32.36	-0.92
6 ½ c.c. serum	21.18 21.00	21.09	4.0	23.78 23.78 25.34	24.30	25.09	-3.1
			8.0	29.44 29.88	29.66	29.09	+1.9
			12.0	33.12 32.12 32.12	32.45	33.09	-1.9
7 ½ c.c. serum	22.18 22.44	22.31	4.0	26.12 26.22	26.17	26.31	-0.53
8 ½ c.c. serum	28.46 28.46 28.46	28.46	4.0	32.50 32.34 32.12	32.32	32.46	-0.46
9 ⅔ c.c. serum	19.25 19.37	19.31	3.0	21.50 21.75 21.85	21.70	22.31	-1.9
			6.0	24.90 24.90	24.90	25.31	-1.6
			9.0	27.75 27.60	27.68	28.31	-2.2
10 ⅔ c.c. serum	20.60 20.60 20.40	20.53	3.0	23.00 22.60 22.60	22.73	23.53	-3.4
			6.0	26.35 26.40	26.38	26.53	-0.56

*Patient jaundiced. Icteric index 10.6.
†Patient had dinitrophenol.

TABLE II—CONT'D

EXP. NO. AMOUNT SERUM USED	SERUM K (MG./100)		MG. OF K ADDED 100 ML.	TOTAL K OBTAINED		TOTAL CALCULATED	ERROR OF TOTAL (%)
	VALUES	AVG.		VALUES	AVG.		
11 ⅔ c.c. serum	20.40 20.50	20.45	3.0	23.55 23.10	23.33	23.45	-0.51
			6.0	25.25 25.15	25.20	26.45	-4.7
			9.0	28.0 28.25	28.13	29.45	-4.5
12 ½ c.c. serum	25.82 25.66	25.74	4.0	29.12 28.66 28.87	28.87	29.74	-2.9
			8.0	33.22 32.50	32.86	33.74	-2.6
13 ½ c.c. serum	19.66 19.66	19.66	4.0	23.34 23.76	23.55	23.66	-0.46
			8.0	27.44 27.76	27.60	27.66	-0.21

E. *Colorimetry.*

1. To a 100 c.c. volumetric flask, add
   2 c.c. of supernatant fluid from D,
   5 c.c. of distilled water,
   1 c.c. of 50% hydrochloric acid, and
   2 c.c. of 0.5% sulfanilamide.
2. Mix and let stand for three minutes, then add 1 c.c. of 0.1% N-1 naphthylethylenediamine dihydrochloride.
3. Make to volume and read after five minutes.

F. *Reagents.*

1. 1.5% sodium tungstate.
2. 2.5% copper sulfate.
3. 2.5% silver nitrate.
4. 0.2 N (approx.) sodium hydroxide.
5. 40% silver nitrate.
6. Sodium Cobaltinitrite Solution.[5]
   A. Dissolve 25 Gm. of cobalt nitrate crystals in 50 c.c. of water and add 12.5 c.c. of glacial acetic acid.
   B. Dissolve 120 Gm. of sodium nitrite in 180 c.c. of water (must be potassium free). Add 210 c.c. of B to all of A, and aerate the solution under the hood until all the nitrous oxide fumes are driven off. Place in the refrigerator where it will keep for about a month. *Filter each time before using.*
7. Cobaltinitrite Reagent.
   To 20 c.c. of filtered sodium cobaltinitrite solution, add 1 c.c. of 40% silver nitrate.
   Shake well and filter to remove trace of precipitate which is undissolved.
8. Sulfanilamide Reagent.
   0.5% sulfanilamide in 30% acetic acid. Must be prepared fresh weekly.

9. Coupler Reagent.
   0.1% N-1 naphthylethylenediamine dihydrochloride in 30% acetic acid. Must be prepared fresh weekly.
10. Wash Reagent.
   2 volumes alcohol.
   1 volume ether.
   2 volumes water.

A standard curve was established by carrying out the foregoing procedure on standard potassium sulfate solutions containing 0.01 to 0.12 mg. of potassium per sample. The extended readings were obtained with a Lange photoelectric colorimeter against a water blank using a green filter having maximum transmission at 5,200 A.U. The results for values up to 0.05 mg. per sample are plotted in Fig. 2. As only one-fifth of the digested cobaltinitrite is taken for the final colorimetric reading, the absolute value of the potassium determined ranges from 0.002 to 0.010 mg.

It will be noted that the line which passes through the mean value for each concentration passes through the origin and follows Beer's law without deviation. Above 0.05 mg. of potassium per original sample the curve falls off sharply. This may be seen in Table I where the extinction constant K was calculated according to the formula $K = \frac{E}{CT}$, where E equals the extinction, C = the concentration of potassium in milligrams per 100 c.c. of final volume, and T = the thickness of the solution through which the light must pass, which, in our tubes, is 1.2 cm.

In the usual procedure the concentration of the potassium in the final solution of the standard is 0.012 mg. and the readings are made at a point at which the color does not follow Beer's law.

Our method, therefore, has been adjusted to give readings corresponding to 0.006 mg. of potassium in the final solution. This is in the region in which there is no deviation from Beer's law and allows determinations to be made on serums containing up to 35 mg. of potassium per 100 ml. without dilution. This would cover the entire range which might be encountered in ordinary clinical practice.

Having established our table of values for known potassium, a series of blood serums were run to which varying amounts of potassium were added. The recovery values may be seen in Table II. It is interesting to note that, although different serums were used in each experiment, there is no wide variation in the potassium value for the serum alone. Cases 8 and 12 had an undetermined amount of potassium added before the known amount was added, which accounts for the high initial values in these two cases.

It will be noted that the determinations made in multiplicate on the original serum show a mean difference from the average value of each serum of only 0.15 mg., and this value is elevated somewhat by the poor result obtained in Case 5 where a difference of 1.78 mg. between the highest and lowest readings was found. If we exclude this case, the maximum difference between the highest and lowest readings of any serum was 0.56 mg. The mean error in the recovery of the added potassium was 1.89 per cent.

SUMMARY

A modification of the silver cobaltinitrite method for the determination of serum potassium adapted for use with a photoelectric colorimeter is described. By the use of sulfanilamide and N-1 naphthylethylenediamine dihydrochloride to determine the concentration of nitrite in the potassium silver cobaltinitrite precipitate a stable color is produced which is sensitive to 0.002 mg. of potassium in 100 ml. of solution. The color produced follows Beer's law up to a concentration of 0.01 mg. of potassium in the final solution, which would correspond to a serum potassium value of 33.3 mg. per 100 ml.

REFERENCES

1. Breh F., and Gaebler, O. H.: The Determination of Potassium in Blood Serum, J. Biol. Chem. 87: 81, 1930.
2. Taylor, F. H. L.: The Determination of Potassium in Blood Serum, J. Biol. Chem. 87: 27, 1930.
3. Truzkowski, R., and Zwemer, R. L.: Cortico-adrenal Insufficiency and Potassium Metabolism. I. Determination of Potassium in Small Quantities of Blood and Tissues. Biochem. J. 30: 1345, 1936; Determination of Blood Potassium, Biochem. J. 31: 229, 1937.
4. Robinson, R. J., and Putnam, G. L.: Determination of Small Amounts of Potassium by Means of Silver Cobaltinitrite, Indust. & Engin. Chem. Analyt. Ed. 8: 211, 1936.
5. Harris, J. E.: A Modified Silver Cobaltinitrite Method for Potassium Determination, J. Biol. Chem. 136: 619, 1940.
6. Weichselbaum, T. E., Somogyi, M., and Rusk, H. A.: A Method for the Determination of Small Amounts of Potassium, J. Biol. Chem. 132: 343, 1940.
7. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. 128: 537, 1939.
8. Shinn, Martha B.: Colorimetric Method for Determination of Nitrite, Indust. & Engin. Chem. Analyt. Ed. 13: 33, 1941.
9. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry-Methods, Baltimore, 1932, Williams & Wilkins Co.

THE MICRODETERMINATION OF CHLORIDES IN VISCOUS BIOLOGICAL FLUIDS AND ITS RELIABILITY*

FRANKLIN HOLLANDER, PH.D., AND JULIUS STEIN, B.S.
NEW YORK, N. Y.

THE need for a simple, rapid, and reliable method for determining micro quantities of chloride in 0.1 ml. samples of biological fluids like blood and viscous mucus is widely recognized, but no procedure which satisfies all these requirements is as yet available. Some adaptation of the Volhard titration (e.g., the method of Van Slyke[16]) would seem to be eminently suitable for the purpose, were it not that the precision of the silver thiocyanate titration is so poor as to rule this technique out of consideration for quantities of chlorine as low as 100 γ. In spite of the popularity of the Wilson and Ball[19] modification of the Van Slyke method for quantities around 3 to 5 mg., occasional articles which call attention to its poor reliability are still being published (e.g., Schales and Schales[12]). The chief cause of this inadequacy is the transience of the end point, which results from the dissociation of the ferric thiocyanate into its colorless constituents, aided

*From the Laboratories of the Mount Sinai Hospital, New York City.
This work was supported in part by a grant from the Friedsam Foundation.
Received for publication, June 25, 1942.

by the conversion of precipitated silver chloride to silver thiocyanate in consequence of the greater insolubility of the latter. Rosanoff and Hill[16] reported that this difficulty in the Volhard titration can be overcome in part by filtering off the silver chloride before titrating the excess silver with thiocyanate, but scrutiny of their data leaves some doubt regarding the relative magnitudes of the errors with and without such filtration. In order to save the time and labor of filtration, Keys[5] adopted the technique of Fiske and Sokhey,[2] and centrifuged before titrating in order to remove the silver chloride precipitate by packing it at the bottom of the titration tube. However, Kolthoff[7] has pointed out that separation of the silver chloride entails the removal of silver-ion by adsorption, and Whitehorn[17] found no improvement whatever as a result of such filtration. These reports led to the use of various chemical agents for reducing the transience of the end point. Smirk[14] reported that acetone in conjunction with alcoholic thiocyanate sharpens the end point by decreasing the dissociation of the ferric thiocyanate, but this effect was not confirmed by Wilson and Ball.[19] The use of nitrobenzene for the same purpose was advocated by Caldwell and Moyer[1]; and Keys[6] employed ether to collect the silver precipitate at the liquid-liquid interface and thus to reduce back reactions in the water phase, according to the principle of Rothmund and Burgstaller.[11] Toluene and benzene have been recommended by Stschigol[15] for the same purpose, but none of these techniques has as yet been generally adopted.

The method of Wilson and Ball has been in use in this laboratory for many years, but we also have never been satisfied with its reliability. Multiple titrations on the same solution are often in very poor agreement, particularly when they are done on different days. The magnitude of these errors is well illustrated by the two series of data for a set of 24 single determinations on the same solution, performed over a period of eight weeks (experiment G 33-9). For the titration of the blanks (2 ml. of 0.2 N silver nitrate, titrated with about 0.5 N potassium thiocyanate) the standard deviation of the entire distribution (s) was 0.0017 meq.* and the mean (M) was 0.394 meq. For the corresponding "unknown" titrations (2 ml. of 0.2 N silver nitrate + 0.5 ml. of 0.1 N sodium chloride) $s = 0.0015$ meq. and $M = 0.344$ meq. Hence, for the 24 titers of the sodium chloride, alone, calculated by difference of blank and unknown, $M = 0.050$ meq. and $s = \sqrt{(0.0017)^2 + (0.0015)^2} = 0.0023$ meq., or 4.6 per cent of the mean (see Addenda, especially equations 4 and 7). Two other sets of similar data (G 33-7 and 8) were in statistically reasonable agreement with these values, and they are all well within the experience of other laboratories in the use of such modifications of the Volhard method. These results, therefore, afford a fair measure of the poor precision of this method as ordinarily carried out. Since these titers were not correlated with the date of titration, there is no reason for suspecting a progressive change in concentration of any of the reagents on standing, and this is supported by the identity of the mean titers for sodium chloride in all three sets of data. Attempts to correlate the error with color intensity of the end point or with individual operator have been unsuccessful.

*All titers and standard deviations deri[illegible] reported in milliequivalents instead of milliliters of potassium thiocyan[illegible] acilitate the comparison of observations on different unknowns, and of [illegible] of different concentrations or volumes.

The afore-mentioned titrations were all performed in the presence of nitrobenzene, since without this substance (or with ether or acetone) the end point is even less reliable than with it. According to Caldwell and Moyer, the efficacy of nitrobenzene depends on its formation of "an insoluble layer over the precipitate, so that the rate of solution of the silver chloride is reduced to such an extent that it does not interfere in the thiocyanate titration," but a perfectly clear supernatant fluid is not essential. In our experience the silver precipitate appears to be removed by suspension within the layer of nitrobenzene, and this process rarely occurs completely. The action of the nitrobenzene can be reversed by the addition of more silver nitrate after the end point has been reached, and this explains why the removal of precipitate occurs only as one approaches the end point where the silver ion concentration is low. In our efforts to reduce the variability of our results we noticed that the uniformity of the titers increased with the completeness of removal of the precipitated silver salts by the nitrobenzene. Furthermore, the completeness of separation of the precipitate from the aqueous layer seemed to increase with the duration and vigor of shaking after each addition of reagent. Caldwell and Moyer state that agitation for thirty to forty seconds is necessary in order to form large spongy flakes, but they apparently did not associate such formation with the retention of these flakes within the nitrobenzene layer. Accordingly, we performed four series of titrations (G 33-10, 11, 12, and 13) by essentially the same procedure as before, except that additional precautions were taken in respect to the vigor and duration of shaking. The results showed marked and consistent improvement over those obtained without this innovation in technique. Thus, a pooled estimate of s (see Addenda, equations 8, 9, and 10) from all 6 sets of data *without systematic agitation* ($N = 170$) is 0.0016 meq., whereas for the 8 such sets on blanks and sodium chloride solutions *with active shaking* ($N = 290$), s is 0.0004 meq. This indicates a fourfold improvement in the precision—a result which was confirmed by similar titrations on standard hydrochloric acid solutions and oxalated whole blood (G 33-10, 12, and 13).

Our experience with the shaking technique has been so consistently good that there can be no doubt regarding the reason for the improvement in reproducibility of titration; i.e., *the systematic removal of silver precipitate from suspension in the aqueous phase by means of vigorous shaking with nitrobenzene.* Nitrobenzene has not always been efficient in yielding good agreement among multiple titrations, but this has resulted merely from the lack of adequate shaking of the titration mixture. The influence of stirring upon the rate of fading of the end point and, therefore, upon the titer, in the absence of nitrobenzene, was pointed out by Short and Gellis,[13] but Whitehorn[19] subsequently reported identical results with mild and with vigorous stirring. In consequence, the importance of intensive agitation in the Volhard titration has been generally neglected (Keys[5] does stir the titration mixture, by means of a jet of air, but only to insure uniformity of titration rate). However, our own observations leave no doubt of its importance for optimum reproducibility of the end point, particularly in view of the large number of titrations used for the estimation of the precision measures.

We have, therefore, adapted this technique to the determination of minute quantities of chlorine (as low as 90γ), and our results have been entirely consistent with expectations based on our experiences with the larger quantities. In order that the procedure may be applied to biological liquids of high viscosity (like mucous secretions) without loss of precision, we have modified the usual pipetting technique—but this may be disregarded for fluids of low viscosity.

PROCEDURE

Reagents.—Silver nitrate (about 0.2 normal, not standardized), sodium chloride (0.1 normal, standardized gravimetrically, $s_M = 0.0004$ N), potassium thiocyanate (about 0.01 normal, not standardized*), ferric alum (6 per cent), nitrobenzene, and concentrated nitric acid.

The procedure is as follows:

1. Pipette 0.1 ml. of silver nitrate into each of a series of pyrex test tubes, 200 mm. by 24 mm. (outside diameter); designate 2 of these as "blanks."

2. Into each of 2 other tubes containing silver nitrate pipette 0.1 ml. of sodium chloride solution; these tubes are designated "NaCl standards" and serve in effect for the standardization of the potassium thiocyanate reagent.

3. Into each of another set of 2 or 3 tubes containing silver nitrate, introduce 0.1 ml. of the unknown solution, using the same pipette† for unknowns as for sodium chloride standards. With viscous liquids rinse the pipette twice with nitric acid and once with water—in which case the sodium chloride standard must also be delivered with rinsing.

4. To each tube add 1 ml. of nitric acid, less whatever may have been employed for rinsing. Cover the tube with a small watch glass and digest the contents on a water bath until the liquid becomes clear; this usually requires from one to two hours. Blanks and controls need not be digested, since their titers are the same with and without heating. Solutions brought to this stage can be stored in the dark for several days, or in dim diffuse light for one day, without any appreciable difference in titer.

5. After removing the tubes from the steam bath, cool and add 0.5 ml. of ferric alum and 6 drops (about 0.2 ml.) of nitrobenzene to each.

6. Titrate with the thiocyanate solution, using a 10 ml. burette graduated in 0.02 ml. A burette tip consisting of a hypodermic needle fitted to a Luer adaptor and the adjustable drop control device previously described (Hollander[3]) permit the addition of small volumes of reagent (0.003 ml.) with a minimum of time and effort. Add the thiocyanate slowly with frequent vigorous shaking, until the precipitate conglomerates into large flakes and is taken up by the nitrobenzene at the bottom of the tube. Slight opalescence in the supernatant fluid, caused by droplets of nitrobenzene, does not interfere with the end point nor reduce the precision of the method. As the end point is approached, shake the titration mixture vigorously after the addition of each drop of thiocyanate, for it is of the utmost importance that the precipitate be taken up entirely by the nitrobenzene at this stage. The nitrobenzene layer is broken up by this

*This solution is prepared with merthiolate (1:25,000) and then filtered to prevent the growth of organisms.

†These pipettes need not be calibrated, for reasons which are given later in this section. To facilitate their manipulation, without sucking the viscous specimen up above the graduation mark, we employ a special mechanical manipulator described elsewhere (Hollander and Stein[4]).

procedure, but it re-forms each time into an opaque spheroidal mass. The end point is indicated by the first faint orange-red tint of the supernatant liquid; under these conditions it is invariably stable for ten minutes and usually for much longer.

7. The concentration of chloride in the unknown, $[Cl]_{unk}$, is calculated by means of equation (2), in which the symbols have the following meanings: v = volume in milliliters of thiocyanate reagent required for unknown, blank, or sodium chloride standard as indicated by the subscript; ml = volume in milliliters of sample pipetted out; $[Cl]_{NaCl}$ = chlorine concentration of the standard sodium chloride solution.

$$[Cl]_{unk} = [Cl]_{NaCl}\left(\frac{v_{blank} - v_{unk}}{v_{blank} - v_{NaCl}}\right)\left(\frac{ml_{NaCl}}{ml_{unk}}\right) \qquad (1)$$

Since the same pipette is used for unknown and for standard solutions, the last factor on the right becomes unity. Then, representing $(v_{blank} - v_{unk})$ by Δv_{unk} and $(v_{blank} - v_{NaCl})$ by Δv_{NaCl}, we have

$$[Cl]_{unk} = \Delta v_{unk}\,\frac{[Cl]_{NaCl}}{\Delta v_{NaCl}} \qquad (2)$$

The expression $[Cl]_{NaCl}/\Delta v_{NaCl}$ is a constant factor for any set of reagents, and this greatly simplifies the calculations required for a long series of analyses.

Van Slyke applied a negative "empirical" correction to each titration reading, because an excess of 0.04 ml. of reagent was necessary to give a decided end point. Keys stated that any method which omits separation of the precipitate from the titrated fluid yields titers which are slightly too high; this error might also be obviated by a correction term similar to Van Slyke's. The titers which we obtained without the effective transfer of the silver precipitate to the nitrobenzene layer were significantly higher than the values obtained by means of vigorous shaking, and this is qualitatively in agreement with the observations of Keys. However, our method of calculation obviates the use of any such correction term, because it would occur twice in the calculation of each Δ v-value and, therefore, cancel out completely.

It will be noted also that the foregoing procedure obviates the independent macrostandardization of the potassium thiocyanate solution, since the same end is attained by titration of the sodium chloride standard. The constancy of concentration of the reagent over a period of several months is demonstrated by the small scatter (s) of the data for blanks and sodium chloride standards throughout any one series of titrations, and, therefore, the titrations of blank and sodium chloride standards need be done infrequently. In view of the trouble commonly encountered with deterioration of thiocyanate solutions, this constancy of its concentration is particularly noteworthy, and we ascribe it to the preparation of the reagent with merthiolate and filtration.

RELIABILITY OF THE METHOD

The reliability of an analytical procedure may be evaluated from the point of view of its accuracy (i.e., agreement between estimated and actual values; correctness) or of its precision (i.e., agreement among different estimates of the same value; reproducibility). In the following discussion the reliability has been considered from both of these angles.

As a measure of the *precision* of the method we performed a set of determinations on each of several solutions: standard hydrochloric acid in different concentrations, gastric mucous secretion, stomach contents, and oxalated whole blood. The number of titrations comprising each series and the statistical data are presented in Table I. In all but one of these series, the individual titrations were performed at various times throughout an interval of five weeks or more. The quantities of chlorine contained in the 0.1 ml. samples of the unknowns varied from 91 γ to 439 γ, corresponding to concentrations of 26 to 124 mN.

Now, in calculating the chlorine concentration of any solution, we actually employ three different titers: for blank, for sodium chloride standard, and for the unknown itself. For the first two of these, we ordinarily use mean values obtained from a large number of titrations (e.g., series 3a and 3b); for the unknown, however, we usually employ a mean of two, or at most three, titrations. Referring to equation (1), we see that s_M for $[Cl]_{unk}$ must be a function of the standard error of all three of these averages—v_{blank}, v_{NaCl}, and v_{unk}. But, since N for the two former is large compared with N for the unknown the s_M values for the former are negligible with respect to s_M for v_{unk} (see equation 5). Consequently, s_M for a Cl determination in triplicate may be taken equal to s_M for the titer of the unknown alone, and this is equal to $s/\sqrt{3}$, where s is the standard deviation of the titration itself. From the data in the table it appears that s varies from 0.00009 to 0.00016 meq., and that these values are not correlated with the mean titer. Hence, the most representative estimate of s for a titration is given by a pooled estimate* made from all 8 series, with a total of 81 individual titrations (N); by equation (11) this possesses the value 0.00010 meq. Therefore, $s_M = s/\sqrt{3} = 0.00006$ meq., and this is the precision measure for a titration done by this method in triplicate.

It must be remembered that the standard deviation observed for any other set of determinations may differ from this population estimate by amounts within the usual limits of statistical variation. Because of the very small number of titrations employed for any one determination, these differences may be relatively large. Furthermore, a percentage evaluation of precision (by means of the coefficient of variation, V) will vary with the magnitude of M according to equation (6). Thus, for a determination on 100 γ of chlorine (0.0028 meq.), V = 2.1 per cent, whereas for 0.5 mg. V is one-fifth of this, or 0.4 per cent. In general, therefore, the standard error in milliequivalents affords a more significant precision measure for an analytical method than does the coefficient of variation.

High viscosity of the unknown specimen does not affect the reliability of the method. This is shown by comparing s for determinations on mucus, blood, and stomach contents (series 6, 5, and 14) with the values for inorganic solutions (series 3a-3c). The good agreement among these statistics results in great measure from our practice of delivering the sample for analysis by rinsing the pipette and our precaution not to draw the sample up above the graduation in the first place.

*The chi-square test for homogeneity of the variances (s^2) justifies the taking of such a pooled estimate.

An estimate of the *accuracy* of this method can be obtained by comparing its results with those determined by a macromethod on the same solution. For this purpose, three solutions of hydrochloric acid were prepared from a carefully standardized stock solution, the concentration of which was 100 normal by titration with sodium hydroxide (which in turn had been standardized against potassium acid phthalate). A gravimetric determination of chlorine in this solution as silver chloride gave a value of 99.9 mN. Based on the mean of these

TABLE I

SUMMARY OF MICROCHLORIDE TITRATION DATA

SERIES	SOLUTIONS TITRATED WITH KCNS (0.01 N)	NUMBER OF TITRATIONS	MEAN TITER* N	±S✦ (MEQ.)	ESTIMATED Cl CONCENTRATION	AMOUNT OF Cl
		N	meq.	meq.	mN	γ
3a (blank)	0.1 ml. $AgNO_3$ (0.2 N)	24	0.0205	0.00009		
3b (standard)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. NaCl (0.1 N)	18	0.0105	0.00009		
----	0.1 ml. NaCl (0.1 N) by difference	-	0.0099			352
3c (unknown)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. HCl (0.1 N)	6	0.0105	0.00012		
----	0.1 ml. HCl (0.1 N) by difference	-	0.0100		99.8	354
3d (unknown)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. HCl (0.05 N)	6	0.0154	0.00011		
----	0.1 ml. HCl (0.05 N) by difference	-	0.0050		50.4	179
3e (unknown)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. HCl (0.025 N)	6	0.0179	0.00016		
----	0.1 ml. HCl (0.025 N) by difference	-	0.0026		25.8	91
6 (unknown)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. mucus†	6	0.0081	0.00013		
----	0.1 ml. mucus by difference	-	0.0124		123.9	439
5 (unknown)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. stomach contents‡	6	0.0111	0.00007		
----	0.1 ml. stomach contents by difference	-	0.0093		93.4	331
14 (unknown)	0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. oxalated whole blood	9	0.0126	0.00007		
----	0.1 ml. oxalated whole blood by difference		0.0075		75.1	266

*Mean amount of potassium thiocyanate used in each titration (in milliequivalents).

†The mucus specimen was collected from a Pavlov pouch in the absence of acid secretion. The sample was extremely viscous and was homogenized before pipetting.

‡This specimen was a sample of fasting contents taken from a dog by means of a Rehfuss tube.

✦s The standard deviation of a single titration.

two values, the three solutions contained the following concentrations and quantities of chlorine: 100.0 mN (354 γ), 50.0 mN (177 γ), and 25.0 mN (89 γ). Six microdeterminations were performed on each of these solutions. The results are summarized in Table I (series 3c, d, and e), and the estimated concentrations are 99.8 mN, 50.4 mN, and 25.8 mN, respectively. These data afford ample evidence of the accuracy of this method.

SUMMARY

1. A considerable improvement in the Van Slyke method for the determination of chlorides in biological fluids can be effected by the addition of nitrobenzene to the titration mixture, as recommended by Caldwell and Moyer, provided the liquid be agitated in such a way as to keep the precipitated silver salts adsorbed to the nitrobenzene layer. The procedure so modified is described in detail.

2. The accuracy and the precision of the method are determined in the range of approximately 100 to 500 micrograms of chlorine (0.1 ml. specimens with a concentration range of 25 to 125 mN). The standard deviation of a single titration, based on a pooled estimate from 81 titrations, is 0.00010 meq., or 1 mN. For a determination done, say, in triplicate, the standard error is 0.00006 meq., or 0.6 mN.

3. This high precision applies to liquids of high viscosity like mucus secretion as well as to hydrochloric acid solutions, provided the samples taken for analysis are pipetted with the proper precautions.

ADDENDA

1. Let

A_i = any one of a series of titers,
N = the number of titers in the series,
M = the mean of this series.

Then, the standard deviation for the series (and hence for a single titration) is given by s in the following equation:

$$s^2 \text{ (or } s_A^2) = \frac{\Sigma(M - A_i)^2}{N} \qquad (3)$$

For the performance of various "tests of significance," it is customary to adjust s^2 with the factor $N/(N-1)$, particularly when $N \leqslant 25$, in order to obtain an estimate of the population variance from that for a sample. This yields:

$$s^2 = \frac{\Sigma(M - A_i)^2}{(N - 1)} \qquad (4)$$

In the present work we are concerned only with a description of errors and not with tests of significance, but we have made this adjustment as a matter of routine. Then the standard deviation of the mean (i.e., the standard error) is given by s_M:

$$s_M^2 = \frac{s^2}{N} = \frac{\Sigma(M - A_i)^2}{N(N - 1)} \qquad (5)$$

and the corresponding percentage value, the coefficient of variation, is given by

$$V = \frac{100 \times s_M}{M} \text{ per cent} \qquad (6)$$

2. For a series of N values, each of which is the difference of two such titers $(A_i - B_i)$, e.g., on blank and unknown solution, respectively, we have

$$s^2_{(A-B)} = S_A^2 + s_B^2 \qquad (7)$$

provided $N_A = N_B$ or that they differ by an amount which is negligible.

3. Given p series of data, each comprising N_1, N_2, --------, N_p titrations, respectively, and each possessing a mean and standard deviation $(M_1 \pm s_1)$, $(M_2 \pm s_2)$, -------- $(M_p \pm s_p)$, respectively. Then, for a pool of all the data we can calculate the same statistics by the following equations:

$$N = \sum_{i=1}^{p} N_i \qquad (8)$$

$$M = \frac{\sum_{i=1}^{p}(N_i \times M_i)}{N} \qquad (9)$$

$$s^2 = \frac{\sum_{i=1}^{p}(N_i s_i^2) + \sum_{i=1}^{p}(N_i d_i^2)}{N} \qquad (10)$$

where $d_i = M_i - M$. If the several M_i values are in fairly good agreement, then d_i is negligibly small, and we have

$$s^2 = \frac{\sum_{i=1}^{r}(N_i s_i^2)}{N} \tag{11}$$

For a discussion of the application of statistical methods to problems of chemical analysis, the reader is referred to an article by Power.[8] For a more detailed and rigorous, though elementary, treatment of these methods see Rider.[9]

REFERENCES

1. Caldwell, J. R., and Moyer, H. V.: Determination of Chloride, Indust. & Engin. Chem., Anal. Ed. 7: 38, 1935.
2. Fiske, C. H., and Sokhey, S. S.: Ammonia and Fixed Base Excretion After the Administration of Acid by Various Paths, J. Biol. Chem. 63: 309, 1925.
3. Hollander, F.: An Adjustable Drop-Control for Burettes, Science 73: 45, 1931.
4. Hollander, F., and Stein, J.: A Mechanical Manipulator for Small Pipettes, J. Lab. & Clin. Med. 27: 1354, 1942.
5. Keys, A.: The Determination of Chlorides With the Highest Accuracy, J. Chem. Soc. 2440, 1931.
6. Keys, A.: The Microdetermination of Chlorides in Biological Materials. Presentation of a Method and an Analysis of Its Use, J. Biol. Chem. 119: 389, 1937.
7. Kolthoff, I. M.: Volumetric Analysis, New York, 1928, John Wiley & Sons.
8. Power, F. W.: Accuracy and Precision of Microanalytical Determination of Carbon and Hydrogen (A Statistical Study), Indust. & Engin. Chem., Anal. Ed. 11: 660, 1939.
9. Rider, P. R.: Statistical Methods, New York, 1939, John Wiley & Sons.
10. Rosanoff, M. A., and Hill, A. C.: A Necessary Modification of Volhard's Method for the Determination of Chlorides, J. Am. Chem. Soc. 29: 269, 1907.
11. Rothmund, V., and Burgstaller, A.: Accuracy of the Volhard Chlorine Titration, Ztschr. anorg. Chem. 63: 330, 1909.
12. Schales, O., and Schales, S. S.: A Simple and Accurate Method for the Determination of Chloride in Biological Fluids, J. Biol. Chem. 140: 879, 1941.
13. Short, J. J., and Gellis, A. D.: Blood Chloride Methods, J. Biol. Chem. 73: 219, 1927.
14. Smirk, F. H.: The Micro-estimation of Chloride in Whole Blood Serum or Corpuscles, Biochem. J. 21: 31, 1927.
15. Stschigol, M. B.: Determination of Chloride by the Volhard Method, Ztschr. f. anal. Chem. 91: 182, 1932.
16. Van Slyke, D. D.: The Determination of Chlorides in Blood and Tissues, J. Biol. Chem. 58: 523, 1923.
17. Whitehorn, J. C.: A System of Blood Analysis. Supplement II. Simplified Method for the Determination of Chlorides in Blood or Plasma, J. Biol. Chem. 45: 449, 1921.
18. Whitehorn, J. C.: Concerning Chloride Determinations by the Modified Volhard Titration, J. Biol. Chem. 74: 299, 1927.
19. Wilson, D. W., and Ball, E. G.: A Study of the Estimation of Chloride in Blood and Serum, J. Biol. Chem. 79: 221, 1928.

A SIMPLE AND RELIABLE METHOD FOR THE DETERMINATION OF METHYL ALCOHOL AND FORMALDEHYDE IN THE AIR*

C. F. Ackerbauer, B.S., and R. J. Lebowich, M.D., Gloversville, N. Y.

IN THE COURSE of an investigation upon the clinical effects of methylation in workers engaged in the tanning industry, it became necessary to determine accurately the percentage of formaldehyde and methyl alcohol vapors in the air. In this paper, the word "methylation" is used to indicate a direct union or substitution of the methyl radical in certain chemical compounds in the tissue cells and body fluids. Nearly all the chemical methods which have been proposed for their estimation have been based upon the principle of a solution of these gases in water or some organic solvent.

The method submitted was developed to substantiate the cause of possible injurious effects found clinically in workers in industries employing formaldehyde or its derivatives and methyl alcohol. In recent years the use of these products has increased tremendously due to their employment in the manufacture of plastics, disinfectants, adhesives, textiles, rubber, and paper.

A critical and exhaustive survey of the literature failed to reveal the existence of any technical method for the accurate estimation of formaldehyde and methyl alcohol in the air, the necessity of which is patent.

Zhitkova[2] in his article on the determination of poisonous vapors in the atmosphere, mentions a method for the detection of these gases. These procedures have been carefully followed, checked and discarded because they did not yield reproducible results with a high percentage of accuracy.

Wright[1] investigated numerous proposed tests for the determination of methanol depending upon its oxidation to formaldehyde. He states: "As oxidizing agents hydrogen peroxide, chromic acid, ammonium persulphate, hot copper spiral and potassium permanganate were tried, but all except potassium permanganate were rejected as unsatisfactory." These findings have been confirmed by us. Rosaniline, identical with Schiff's reagent modified, proved to be the most sensitive and stable reagent and yielded the most reliable and reproducible results. It was necessary to modify this method slightly in order to differentiate between formaldehyde vapors and those of methyl alcohol. The commercial use of formaldehyde fully embraces the use of methyl alcohol because, as manufactured at present, it contains 1 to 8 per cent of methyl alcohol, according to specifications, in order to prevent the possible polymerization of formaldehyde to paraformaldehyde.

Adaptation of the modified Schiff's reagent required the forced solution of formaldehyde and methyl alcohol vapors into an aqueous solution, eliminating other fumes which might cause interfering chemical reactions, thereby markedly invalidating the accuracy of the method. In the field, chlorine, sulfur dioxide,

---

*From the Eugene Littauer Memorial and Fulton County Laboratory, Nathan Littauer Hospital, Gloversville.

Received for publication, June 26, 1942.

formic and acetic acid fumes reacted with the reagents in the receivers, destroying the formaldehyde condensation violet color formed by the union of rosaniline and formaldehyde. Therefore, it was essential to develop an apparatus for the absorption of these gases from the air.

The method satisfied the following criteria: (1) Complete absorption of the vapors of formaldehyde and methyl alcohol present in a measured volume of air with their negligible condensation upon the walls of the vessels containing the absorbing fluids. (2) Regulation of their rate of diffusion and absorption. (3) Complete elimination of interfering and extraneous gases.

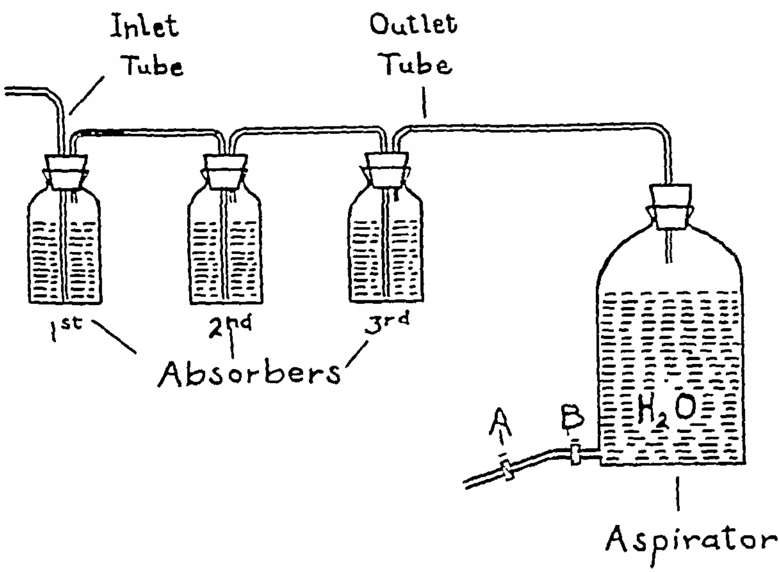


Fig. 1.—*A*, Screw clamp (snap). *B*, Screw clamp (variable).

DESCRIPTION OF APPARATUS AND ABSORBING SOLUTIONS

For routine analysis an apparatus was constructed according to Fig. 1. The gas absorbers consist of three stock laboratory bottles fitted tightly with a two-holed rubber stopper. One hole of each stopper accommodates a ¼ inch diameter glass inlet tube extending to within ⅛ inch from the center of the bottom of the bottles. The other hole accommodates a ¼ inch diameter glass outlet tube which extends ¼ to ½ inch below the bottom of the stopper.

The bottles are so arranged in a series of three that the first inlet tube is joined to a glass tube of ½ inch diameter. The last outlet tube is connected to a 5 liter glass aspirating flask, the outlet of which regulates the flow and volume of the test sample of air through the system. Two screw clamps serve as valves to adjust the flow of water from the aspirator; one is permanently set to insure the same rate of flow for successive determinations; the other acts as a shut-off for the entire system. The diameters of the inlet openings are adjusted to a 1.6 mm. aperture to allow only small bubbles of the incoming vapors to pass through the liquids in the absorbers in a steadily flowing stream. Larger openings would permit the passage of excessively large bubbles carrying traces of impurities of sulfur dioxide, chlorine, and carbon dioxide, and these represent a source of considerable technical error. The use

of aerator stones was investigated and determinations with them proved inaccurate. They introduced a phenomenon of selective adsorption which did not remain constant throughout the period of time necessary to conduct a test.

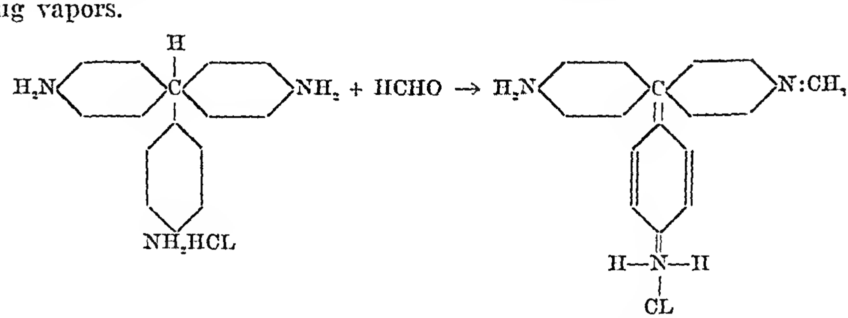
After many experiments the following solutions and their indicated concentrations were selected for the absorption of the interfering vapors and those to be estimated.

The first absorber containing 150 c.c. of a 1 per cent phosphoric acid and 2 per cent barium chloride solution in equal amounts removes all the sulfur dioxide fumes and the formic and acetic acid vapors. Frequent check tests were made on this solution to determine the amount of formaldehyde and methyl alcohol vapors absorbed, and the results revealed their presence only in minute negligible traces. This fact is believed to be due to the property of formaldehyde and methyl alcohol being more unstable in an acid solution than in an alkaline solution. The mild state of aeration tends to influence the presence of formaldehyde and methyl alcohol as a gas. The carbon dioxide, as it passes through water, forms carbonic acid, which reacts according to the following equation. The phosphoric acid acts as a carbonic anhydrase, as first purified by Meldrum and Roughton.[3] This accelerates the reaction in both directions: $H_2CO_3 + BaCl_2 \leftrightarrows 2HCl + BaCO_3\downarrow$.

The second absorber contains 200 c.c. of an alkaline solution of 5 per cent potassium permanganate, which completely removes by oxidation all the methyl alcohol.

The third absorber contains 225 c.c. of modified Schiff's reagent, and the fourth, employed for experimental purposes, holds 200 c.c. 2 N sodium bisulfite solution.

The errors introduced by the entrainment of formaldehyde vapors into the first and second absorbers were found to be 1 part in 1,000 in the first absorber and 3 parts in 1,000 in the second absorber. In the third absorber, over 97 per cent of the formaldehyde vapors react with the modified Schiff's reagent, according to the following supposed equation forming the characteristic Schiff's violet color,[1] the intensity of which is proportional to the amount of reacting vapors.



The fourth experimental absorber, although unnecessary, was used to detect any escaping formaldehyde vapors by their reaction with sodium bisulfite solution, forming oxymethyl sodium sulfonate, according to the equation:

$$HCHO + NaHSO_3 \rightarrow H_2C\begin{cases} OH \\ SO_3Na \end{cases}$$

In some instances, volumes of vapors as high as 3.5 per cent were noted under conditions where the temperature of the surrounding atmosphere was above 80° F.

DESCRIPTION AND RATIONAL OF METHOD

By means of the variable screw clamp, the vacuum created by the flow of water is regulated so that one liter of water flows freely without pulsations every twenty-five minutes. It is absolutely necessary that pulsations be eliminated, because many large bubbles may be forced spasmodically through the absorbers so rapidly that they may carry with them entrained vapors which are not absorbed in their respective solutions.

In order to absorb completely all the formaldehyde and methyl alcohol vapors, as well as to remove all interfering gaseous substances, it is necessary to allow at least 1 liter of water to flow from the aspirator. By experimentation with the object of reducing the percentage of technical error in the method to a minimum, it was found that the use of 5 liters of water yields very accurate results, but a volume of 5 to 10 liters is even more reliable and, therefore, desirable, as shown in Table II.

In a routine analysis, 1,000 c.c. of vapors are allowed to pass through the system in order to remove the air above the surface of the liquid in each absorber and saturate it with the vapors to be expelled from that solution. One liter of water is permitted to flow from the aspirator for a period of twenty-five minutes, and then its flow is stopped by the second snap clamp. The level of water is again adjusted in the aspirator and another 1,000 c.c. of air are inducted. While the aspirator is being filled, a vent must be opened or the liquids in the absorbers will be forced backward through the system, thus invalidating the results. This is repeated until at least 5,000 c.c. of air have been drawn through the receivers.

Absorbers 2 and 3 are removed, sealed with a rubber stopper, and are brought to the laboratory. To repeat the determinations, it is only necessary to renew the liquids in absorbers 2 and 3. The liquid in absorber 1 is sufficiently concentrated to be employed for at least ten determinations without renewal.

In the laboratory, the procedure for the analysis of the solutions in absorbers 2 and 3 is followed according to that outlined by Wright.[1] The potassium permanganate solution is decolorized with the oxalic-sulfuric acid solution and then the modified Schiff's reagent is added. Color comparisons are made with standards already set up and are matched with the naked eye, or preferably with a Hellige colorimeter.

Comparisons were made with the photoelectric colorimeter but were found to be unreliable, due to the inability of the photoelectric cell to differentiate between different wave lengths in the red and blue zones as well as to the intensity of these colors.

If the fourth absorber is used, the per cent of formaldehyde gas is determined volumetrically, using rosolic acid as an indicator, according to the equation:

$$HCHO + Na_2SO_3 + H_2O \rightarrow NaOH + NaSO_3 + (H_2COH)$$

In the field it was found that absorber 4 could be eliminated without seriously affecting the results.

Table II shows the results of the experiments conducted in a room, the known composition of its air being assumed to be 14 parts per 100,000 of HCHO and 0.6 parts per 100,000 CH_3OH. This assumption is based upon the fact that the possible condensation and adsorption of the vapors on the walls of floor and ceiling of the rooms and containers are negligible. Another assumption is that the rate of diffusion of these vapors becomes a constant after three to four hours. The tests show the effect of the rate of flow of 1 liter of air through the system in the period of time designated. All tests were conducted with the temperature maintained at 70° F.

TABLE I

TEST	TIME (MINUTES)	ABSORBER 2 CH_3OH (P.P.M.*)	ABSORBER 3 HCHO (P.P.M.)	ABSORBER 4 HCHO (P.P.M.)
1	5	Trace	Trace	4
2	10	Trace	Trace	10
3	15	Trace	10	60
4	20	Trace	50	50
5	25	1	128	10
6†	30	1	140	5

*Pulsations occur because the water did not flow freely but in drops.
†p.p.m. = parts per million.

TABLE II

TEST	VOLUME IN LITERS	ABSORBER 2 (P.P.M.)	ABSORBER 3 (P.P.M.)
1	1	1	130
2	3	1.5	132
3	5	5	140
4	10	7	148
5	12	6	150
6	15	7	149

Intervals of twenty-five minutes were decided upon for the period of the rate of flow. Table II shows the results of the experiments using the rate of flow of 1 liter of air for twenty-five minutes under the same previous existing conditions and with an increase of the volume of air passing through the absorbers before the laboratory tests are performed.

If the larger volumes of air are used, the intensity of color makes comparison with the standards more difficult to match with accuracy. It was also found that some vapors would escape through the aspirator, thus demonstrating that under these conditions the solutions in the absorbers had become saturated.

Table III shows the results of the experiments using the same standard conditions previously stated and varying the height of the inlet tube from the floor.

TABLE III

TEST	HEIGHT OF INLET TUBE FROM FLOOR IN FEET	ABSORBER 2 (P.P.M.)	ABSORBER 3 (P.P.M.)
1	1	20	100
2	3	8	180
3	5.5	3	160
4	7	1	80
5	10	1	30

A sealed room with temperature controlled at 70° F. was used for obtaining results shown in Table III. Vapors were introduced at a point as near the middle of the room as possible. This more closely resembles conditions as found in the field analysis. Although most of the material remain at the floor level, the height where the solutions have a greater tendency to vaporize would be around 5 feet.

These results show the effect of diffusion upon the vapors of CH_3OH and HCHO in the air in a room after three to four hours. The height 5.5 feet was selected as the average height of a person present in a work room. The results of Table III also demonstrate the percentage of vapors in a room at this height where workmen might inhale or come into contact with these vapors.

SUMMARY

A simple and reliable method is submitted for the estimation of methyl alcohol and formaldehyde vapors in the air, employing the modified Schiff's reagent of Wright, and having a possible technical error up to 7 per cent.

REFERENCES

1. Wright, L. O.: Comparison of Various Tests for Methanol, Indust. & Engin. Chem. (Indust. Ed.) 19: 750, 1927.
2. Zhitkova, A. S.: Service to Industry, 1936.
3. Meldrum, N. U., and Roughton, F. J. W.: Carbonic Anhydrase. Its Preparation and Properties, J. Physiol. 80: 113, 1933.

REVIEWS

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

A Manual of Allergy*

THE author explains the writing of this book in the fact that while there are satisfactory large texts on the subject for the use of allergists, and while there are popular books through which the layman can understand allergy, there is need for an intermediate volume which will serve the medical man whose interest is only secondary and who does not wish to burden himself with extensive reading of the larger volumes.

Dr. Cohen's many important contributions to the subject give to his writings a generally recognized authoritativeness.

The book is well written and covers the subject excellently to the extent that general practitioners and nonmedical persons as well can gain a clear understanding of the processes of allergy. One is left with the feeling that Dr. Cohen would have done well to have enlarged it a little more with detailed discussion of the routines of specific diagnosis and treatment. One who has read the book and desires to proceed with allergic studies must still refer to the larger volumes. Although it would be desirable to have it all in one book, there is justification for the author's plan of presentation, in that those who actually wish to use allergic procedures should become acquainted with intricate details as well as with general principles. This certainly should make for better allergic practice.

In this sense we might consider the present volume as an Introduction to Allergy.

An Atlas of Human Anatomy†

TOLDT'S *Atlas of Human Anatomy*, first published in this country in 1919, has gone through two editions and four printings. This no doubt accounts in part at least for the surprisingly reasonable cost of the latest printing. The volumes consist almost exclusively of plates, each of which contains duplicate Latin and English terminologies. Division into two volumes makes for easy handling. Although the sequence of plates is that commonly used in most anatomies, beginning with bones, joints, and muscles, and ending with the nervous system and special senses, the sequence continuity is more logical than in some other texts, facilitating progression from one area to a neighboring one. This is especially illustrated in the muscles of the lower extremities.

Internal Medicine in Old Age‡

INTERNAL MEDICINE IN OLD AGE can be divided roughly into two parts: the first, a short survey of the nature and problems of aging and old age; the second, a comprehensive review of the clinical and diagnostic features and therapeutic management of those

*A Manual of Allergy—For General Practitioners. By Milton B. Cohen, M.D., Director of the Asthma, Hay Fever, and Allergy Foundation; Visiting Physician in Allergy, St. Alexis Hospital, Cleveland, Ohio. Cloth, 156 pages, $2.00. Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers, New York and London, 1941.

†An Atlas of Human Anatomy. For students and physicians. By Carl Toldt, M.D. Assisted by Professor Alois Dalla Rosa, M.D. Adapted to English and American and International Terminologies by M. Eden Paul, M.D. Brux., M.R.C.S., L.R.C.P. Vol. I. New second edition containing sections on Regions of Human Body, Osteology, Arthrology, Myology (Figures 1 to 640, and general index to the two volumes), 390 pages. Vol. II. New second edition containing sections on Splanchnology, Angeiology, Neurology, Organs of the Senses (Figures 641 to 1505, with appendixes and general index to the two valumes.) Cloth, copyright 1919. Reissued in two volumes, June, 1926. Reprinted January, 1941. The Macmillan Company, New York.

‡Internal Medicine in Old Age. By Albert Mueller-Deha ... Physician, ... hronic Disease (Second Divisio ... New York ... Professor of Internal Medicine, ... School, an ... ision, ... S. Milton ... Profe ... School, Col ... ave): Cloth, 396 pages, $5.00. A William Wo ... more, 1942.

diseases most common in the late decades of life, as well as of diseases more common in early years which present special problems in the aged.

The book should be of great value to those who desire an introduction to the field of geriatric medicine, as well as to those practicing physicians who desire ready reference to the pictures presented by disease in the aged. It is simply written and well-documented. The authors' practice of including a liberal number of thumbnail case histories adds interest to the reading and emphasis to the text, underlining the thesis that diagnosis of disease in the aged presents special problems, is not to be undertaken without mature reflection, and is, in spite of all, often mistaken or missed.

Internal Medicine in Old Age offers a wealth of therapeutic suggestions. Nothing seems omitted which might bring relief to the patient. There are valuable suggestions concerning nursing technique as well as chemotherapeutics. The reviewer recommends the book to student and practitioner alike as a valuable and timely review of geriatric medicine.

Clinical Pellagra*

LIKE the period at the end of a paragraph this book punctuates an epoch in the study of pellagra. Dr. Harris starts his discussion with a comprehensive historical review of the early recognition and incidence of the disease, and the early theories as to its causation. This makes interesting reading. Next is a summary of the experimental investigations, those preceding vitamin studies and the vitamin studies themselves.

The major portion of the book is devoted to present knowledge of the etiology, pathology, symptomatology, diagnosis, prognosis, and treatment of the disease. The chapter on Prophylaxis is especially important.

Collaborating contributors from the several institutions, especially in the South, where constructive research has been carried out, have written chapters on the work done in these institutions.

Here we find in a single volume all that need be summarized concerning pellagra.

Laboratory Diagnosis of Protozoan Diseases†

COLONEL CRAIG'S manual of laboratory methods for the diagnosis of diseases caused by protozoan organisms is especially appropriate at the present time when, with American forces in tropical climates, the problem of protozoan infection bids fair to become extremely important. Somewhat over a third of the book is devoted to amebic infection and considerable space is also given to malaria. The remainder of the book deals with the other common tropical infections, as well as protozoan infestations commonly seen in the United States, such as the flagellate infections.

The volume will undoubtedly be widely read.

The Principles of Neurological Surgery‡

THE second edition of Davis' *Neurological Surgery*, like the first, contains full discussion not only of neurological procedure but also of neurologic diagnostic methods. For this reason, it will be useful both to surgeons and to neurologists and internists.

*Clinical Pellagra. By Seale Harris, M.D., Professor Emeritus of Medicine, University of Alabama, Birmingham, Ala. Assisted by Seale Harris, Jr., M.D., formerly Assistant Professor of Medicine, Vanderbilt University, Birmingham, Ala.; with Foreword by E. V. McCollum, Ph.D., Sc.D., LL.D., Professor of Biochemistry, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore, Md. Cloth, illustrated, 494 pages. The C. V. Mosby Company, St. Louis, 1941.

†Laboratory Diagnosis of Protozoan Diseases. By Charles Franklin Craig, M.D., M.A. (Hon.), F.A.C.S., F.A.C.P., Colonel, United States Army Medical Corps, Retired D.S.M. Emeritus Professor of Tropical Medicine, Medical School, Tulane University; Member, American Academy of Tropical Medicine, American Society of Tropical Medicine, American Society of Parasitologists; Honorary Member, American Society of Clinical Pathologists, etc. Cloth, illustrated with 54 engravings and 4 colored plates, 349 pages, $4.50. Lea & Febiger, Philadelphia, 1942.

‡The Principles of Neurological Surgery. By Loyal Davis, M.S., M.D., Ph.D., D.Sc. (Hon.), Professor of Surgery and Chairman of the Division of Surgery, Northwestern University Medical School, Chicago, Ill. Cloth, ed. 2, thoroughly revised with 154 engravings and 298 illustrations and 5 colored plates, 503 pages, $7.00. Lea & Febiger, Philadelphia, 1942.

Biological Symposia*

BIOLOGICAL SYMPOSIA comprises four symposia arranged by the Council of the American Society of Biological Chemists for the Chicago meeting of the Federation in April, 1941. The following subjects of current interest are discussed by authorities in each field:

End Products of Nitrogen Metabolism in Plants; End Products of Nitrogen Metabolism in Animals; Merging of Growth Factors and Vitamins; The Oxidation of Branched-Chain Fatty Acids; The Origin and Regulation of Ketone Bodies from Fats; The Anabolism and Function of the Phospholipids; The Formation of Animal Body Fat. Oxidation Catalysts; Phosphorylation of Glycogen and Glucose; Oxidoreductions in Carbohydrate Breakdown; Pyruvate Oxidation and the Citric Acid Cycle. Choline, the B Vitamins and Fat Metabolism; The Relation of Choline to the Kidneys; The Nature and Significance of Cholinesterase; Interrelationships Between Choline and Other Methylated Compounds.

Textbook of Embryology†

NOT even embryology has reached a static stage in medical science. Jordan and Kindred's fourth edition has quite a number of changes from the third edition. New subjects include embryonic hormones, the *in vitro* development of mammalian embryos, transplantation, and extirpation experiments designed to reveal differential potencies of different portions of the blastoderm, results of administration of growth and sex hormones, and results of restriction of vitamins. There are 78 new illustrations.

The Conquest of Bacteria‡

A "POPULAR" exposition is *The Conquest of Bacteria*, which can be commended for the general reader, especially because, as stated by Sigerist in the Foreword, it tells the story of chemotherapy in simple and sober terms. It is interesting and informative. A safe book for the public.

A Manual of Endocrine Therapy§

THE *Manual of Endocrine Therapy* might at first glance give the impression that the author is unduly optimistic about the results in too wide a variety of maladies. In the Summary of Therapy, Chapter 15, we find such subjects as acne, dysmenorrhea, enuresis, frigidity, and homosexualism, with a listing of the appropriate endocrine medications and dosage. However, when one turns to the body of the small book he finds that Dr. Cinberg's discussion of these and similar subjects is adequately critical. In other words, in the summary he presents the recommendations of others, many of which he himself has found to be unwarranted. This is a handy, quick reference manual for therapy which can be well recommended. It is unfortunate that there has been an error in the index so that in most cases the subject referred to appears on the page following that indicated by the index.

---

*Biological Symposia. Edited by Howard B. Lewis, Professor of Biological Chemistry, University of Michigan. Vol. V, 247 pages. The Jaques Cattell Press, Lancaster, Pa.

†Textbook of Embryology. By Harvey Ernest Jordan, M.A., Ph.D., Sc.D., Professor of Anatomy and Director of the Anatomical Laboratories, University of Virginia; and James Ernest Kindred, M.A., Ph.D., Professor of Anatomy, University of Virginia. Cloth, ed. 4, 613 pages, $6.75. D. Appleton-Century Company, Inc., New York and London, 1942.

‡The Conquest of Bacteria. From Salvarsan to Sulphapyridine. By F. Sherwood Taylor. Foreword by Henry E. Sigerist. Cloth, 178 pages, $2.00. Philosophical Library, New York.

§A Manual of Endocrine Therapy. By Bernard L. Cinberg, B.A., M.D., Diplomate American Board Obstetrics and Gynecology; Lecturer Obstetrics, New York Polyclinic Medical School and Hospital; Adjunct Obstetrician, Beth Israel Hospital, New York City; Gynecologist, Good Samaritan Dispensary. Cloth, 178 pages, $3.25. Chemical Publishing Company, Brooklyn, N. Y., 1942.

The Journal of Laboratory and Clinical Medicine

Vol. 28 | January, 1943 | No. 4

SYMPOSIUM ON WAR MEDICINE. I.

Guest Editorial

THE PROFESSION'S WAR RESPONSIBILITY

Colonel James M. Phalen, Editor, The Military Surgeon

IT WAS with a sharp note of dissatisfaction and of warning to the medical profession of the country that Mr. Paul V. McNutt, Chief of the War Man Power Board, spoke to a session of the American Medical Association at its Atlantic City meeting in June, 1942. In direct and unequivocal terms he stated that the voluntary program for meeting the medical needs of the armed forces and of war industries had been a failure, and that unless the plan produced greatly improved results within a short period "a more vigorous plan," presumably the draft of medical personnel, would have to be put into operation.

Following the statement that the needs of the Army and Navy for medical officers were not being met, he said that it was apparent that the "careful safeguards" that had been set up by the Association's Procurement and Assignment Service had slowed down the rate of recruitment of doctors for the services. If this be indeed so, it is an example of a miscarriage of well-laid plans. The program for procurement and assignment was the result of diligent and well-considered planning by thoroughly competent men. It was calculated to feed medical officers into the armed services as they were needed, to provide skilled attendance to workers in the war industries, and to guarantee that the civilian population should not be deprived of medical attendance. The functions of the agency were of necessity decentralized, the unit of administration being a state board. Why these theoretically perfect plans have not produced the results expected is difficult to say. Mr. McNutt did not elaborate upon his general indictment of the "careful safeguards" of the Association program. The criticism would indicate an undue solicitude for the wants of the civil population.

The speaker charged a marked difference in the enthusiasm of doctors for voluntary service between the war of 1917-1918 and the present conflict. The figures that he gave for voluntary inductions into the services during comparable periods of the two wars, if they could be accepted as wholly valid, would constitute a keen indictment of the present generation of physicians. But conditions were by no means the same and really just comparisons are not possible. There have been, however, very convincing evidences of reluctance of eligible physicians to enter the military services. The lowering in 1940 of the draft age from 34 to 28 years was quickly followed by a marked drop in voluntary inductions of physicians who would otherwise be subject to the draft. The restoration of the former age limits acted as a distinct spur to recruiting.

We cannot question Mr. McNutt's statement that recruitment of the medical profession had not been satisfactory. He said that 5,000 additional doctors were needed before the end of June, 1942, and 20,000 more before the end of the year. These figures give a vivid picture of an acute recruitment problem. The estimate was made that it would take one-third of the physicians of the country to meet the needs of the armed services and that this would embrace two-thirds of those under the age of 45 years.

Dr. Fred W. Rankin, the incoming president of the Association, could only agree with the main items of criticism and that the profession had a definite responsibility in the allotment of its services in this time of emergency. He cited the progress that was being made in bringing back into active practice a great number of retired or semiretired physicians, thus making possible the release for military service of many otherwise indispensable practitioners of younger years.

The basic fact is that there are not enough doctors in the country for essential services. Like sugar and gasoline, they must needs be rationed. The task of this rationing was assigned to the Procurement and Assignment Service, which is now under criticism. The voluntary features of this plan will be followed until they are found definitely lacking. Only then can we look for the rationing to fall into other hands and the "more vigorous plan" put into effect.

THE ROLE OF CONTEMPORARY MEDICINE IN THE CURRENT WAR EFFORT

Lieut. Col. Joseph Rogers Darnall, M.C., U. S. Army

As CIVILIZATION staggers painfully through the kaleidoscopic tragedy of another World War, the characters in this great drama emerge and assume their destined roles. Medicine is honored with an heroic but difficult part in this colossal play, and proper performance is essential if the Axis villain is to be destroyed and the drama is to end happily.

This is by no means the first time that medicine has served humanity by playing a beneficient part in the promulgation of war. Since the days of Moses, the first great sanitarian, civilizations have risen and fallen as a result of the influence of disease upon military campaigns.

While inventive genius throughout the ages has perfected deadlier weapons, so also the minds of men have devised more effective protective measures to preserve the human race. The ever-increasing strength of scientific medicine provides a powerful bulwark against the rising tide of destructive warfare which is sweeping over the world today.

It is not my intention to review the medical accomplishments of former years other than to mention briefly a few of the outstanding achievements since 1914, and then to outline the contributions that are now being made by medicine toward the successful prosecution of the war. Many medical contributions to the current war effort would be impossible were it not for the advances in medicine which occurred during previous military conflicts or as a result of them.

During Kaiser Wilhelm's bloody bid for immortality in those hectic years of 1914-1918, preventive medicine climbed to new heights. Standardized methods of controlling and treating venereal diseases, initiated by the United States Army in 1909, were developed on a large scale in 1917. The undisputed value of typhoid vaccine and of tetanus antitoxin was established beyond doubt as a result of the experience of World War I. The cause of that great louse-borne scourge, trench fever, was discovered, and the control of this important war disease which sapped the fighting strength of great armies was accomplished. Following the Armistice, the determined attack of American medicine on typhus fever in Serbia and Poland demonstrated the usefulness of intensive educational campaigns as a means of controlling communicable disease.

The first World War also stimulated progress in air-conditioning, in rat-proofing, and in aviation medicine. In general, there was an enormously increased dissemination of knowledge concerning hygiene and sanitation, and a healthy expansion of clinical activities in the field of public health following the Armistice of 1918.

Internal medicine gained ground as a result of experience acquired on the battlefields and in the hospitals and laboratories a quarter of a century ago. Extensive investigation of respiratory diseases was stimulated by the pandemic of influenza. As the years unfolded, the laboratory segregated and subdivided the numerous pneumococcus pneumonias. During World War I a therapeutic antitoxic serum was developed to combat the dreaded gas gangrene. The problems and vast experience of that war spurred neuropsychiatry to greater knowledge, and better care for the victims of permanent psychoses was encouraged. Fever therapy of neurosyphilis was inaugurated when the malarial blood of two "shell-shocked" German soldiers was injected into the veins of paretic patients with encouraging results.

"Functional" disorders of the heart and of the neurovascular mechanism became conspicuous during the war. Later, as time relentlessly rolled by, veterans with degenerative cardiovascular disease presented themselves for treatment at government hospitals in ever-increasing numbers, thus focusing attention on those "middle-age" maladies which have made the profession more "coronary conscious."

Little need be said of the well-known contributions of World War I to the advancement of surgery. Battle casualties provided a vast clinical material which resulted in improved surgical knowledge and technique. Mention should be made, nevertheless, of the improved management of orthopedic cases, the Orr method of treating compound fractures, revival of debridement, and development of the Carrel-Dakin technique. The war gave birth to thoracic surgery and produced improved treatment of empyema, shock, osteomyelitis, and septic arthritis, to say nothing of the great gains made in faciomaxillary and plastic surgery, or of improvements in the suturing of blood vessels and nerves.

Physical therapy and occupational therapy were infused with life and became useful adjuncts of orthopedics and neuropsychiatry during and following the Kaiser's war to impose Teutonic Kultur upon an unwilling world.

It cannot be denied that military resistance to German aggression in 1914 also brought contributions in the field of medical organization, education, and literature. The examination of conscripts then, as now, drew public attention to the lack of physical and mental fitness and focused thought on the interrelated problems of medicine, economics, and sociology. The war showed medicine the value of teamwork and logical division of labor, so that specialization developed and group practice flourished when peace came.

Although much may be said concerning the contributions of war to the advancement of medicine, we are at present more vitally concerned with what medicine can contribute to successful prosecution of the war and a United Nations' victory.

With justifiable pride it may be pointed out that the medical departments of our armed services were not caught napping when the Japanese struck at Hawaii and the Philippines on Dec. 7, 1941. Long before that fateful date medical plans had been laid and preparations made to meet such an eventuality.

The advent of selective service training in 1940 signalized important developments in the mobilization of American medicine for war. These develop-

ments may be briefly summarized as follows: Beginning in June, 1940, at the request of the Surgeons General of the Army, Navy, and Public Health Service, the American Medical Association undertook a survey of the physicians of the United States, with special reference to their qualifications and availability. This was accomplished under the direction of the Committee on Medical Preparedness of the American Medical Association.

A total of more than 185,000 physicians received questionnaires from this Committee and by Jan. 2, 1942, more than 158,000, or nearly 86 per cent, had returned them. Considering the average physician's instinctive avoidance of paper work and hostility to questionnaires, this response of the profession was indeed a tribute to the cooperative spirit of American medicine in furthering the war effort.

Thus the foundation was laid for the establishment of the Procurement and Assignment Service for Physicians, Dentists, and Veterinarians on Oct. 30, 1941. This service, which is now under the War Manpower Commission, was established by the President as one of the subdivisions of the Office of Defense Health and Welfare Service. Another subdivision of the Office of Defense Health and Welfare Service is the Health and Medical Committee, which occupied adjoining offices and collaborated closely with the Procurement and Assignment Service. This Health and Medical Committee was first established on Sept. 19, 1940, by the Council of National Defense, "to advise the Council of National Defense regarding the health and medical aspects of National Defense and to coordinate health and medical activities affecting National Defense."

When, on Sept. 3, 1941, the President set up the Office of Defense Health and Welfare Service with Mr. Paul V. McNutt as Director, the Health and Medical Committee was transferred to that office. This committee deals with the more general aspects of medicine's participation in the war effort through its subcommittees on Dentistry, Hospitals, Industrial Health and Medicine, Medical Education, and Negro Health.

One of the first agencies to assist the Surgeons General of the Army, Navy, and Public Health Service in the current war effort was the Division of Medical Sciences of the National Research Council of the National Academy of Sciences. More than 40 committees and subcommittees, covering practically every field of medicine, were organized in 1940. These committees included more than 200 physicians, eminent in their various specialties.

The National Academy of Sciences was established by President Lincoln in 1863, under a Congressional charter which stipulated that the Academy, whenever called upon, should advise the government on any subject of science or art. It is significant that the need for such a scientific advisory body was keenly felt as long ago as the War Between the States.

The National Research Council of the Academy is composed of nine divisions, one of which is the Division of Medical Sciences. No less than 17 national societies of medicine and allied sciences are members of the Division of Medical Sciences.

The Division of Medical Science committees of the National Research Council do not duplicate the activities of the Health and Medical Committee of the

Defense Health and Welfare Service. The latter covers the more general aspects of health and national defense, while the National Research Council committees are called upon, as quasi-governmental agencies, for special professional advice and research.

Anticipating the likelihood of disease epidemics arising as a result of military mobilization, the Surgeon General of the Army, on Dec. 27, 1940, proposed the establishment of a "Board for the investigation and the control of influenza and other epidemic diseases in the Army." The creation of this board was approved by the War Department on Jan. 11, 1941, and the Surgeon General proceeded to organize the board by the assignment of a number of distinguished civilian consultants, each appointed by the Secretary of War.

The board is composed of (1) a central advisory group of seven members and (2) eight investigative commissions concerned primarily with the following eight problems: influenza, meningitis, measles, neurotropic virus diseases, streptococcus diseases, epidemiological surveys, pneumonia, and cross infections.

It may properly be deduced from the afore-mentioned steps to insure collaboration with the best civilian medical talent, that the medical departments of the armed services were keenly alert to the need for medical preparedness. The response of the profession in cooperating with the military by early organization of useful medical agencies to assist the preparedness program has been excellent.

The role of contemporary medicine, however, is not confined to *civilian* participation in the current war effort. It embraces also the organization and activities of the medical departments of our armed forces, which are primarily concerned with furnishing medical service to the Army and Navy. The mission of these medical departments is "to conserve fighting strength," and "to keep as many men at as many guns as many days as possible."

The principal part played by medicine in the first act of the drama of war was the selection of suitable military personnel by excluding from the armed services the physically and mentally unfit. This called for the formulation of appropriate physical standards, and the judicious interpretation of these standards by the thousands of physicians, civilian and military, who were called upon to accept or to reject volunteers for enlistment and selectees for induction.

In the second act of the military drama, medicine plays the part of a guardian angel. In this role, through the application of modern principles of sanitation and disease prevention, medicine protects against pestilence and strives to keep the military personnel, as well as industrial workers, in good physical condition.

In the third act of the war drama, tragedy is the keynote, and medicine appears as the healer of wounds and disease. The unfortunate participants, military and civilian, who become disabled must be furnished with such aid in the form of evacuation and hospitalization as will speedily restore them to health and fighting efficiency, so that the show may go on to the fourth and final act in which medicine assumes another major role in the rehabilitation of the disabled and in the reconstruction and stabilization of civilization. This last act is interminable and the curtain is never run down!

If American medicine is to accomplish its wartime mission, physicians must make great sacrifices as in 1917-1918. Since Pearl Harbor many medical veterans of World War I have developed "itching feet" and have been clamoring for another "hitch" of active duty. Many of them are now in uniform, and others, whose patriotism is unassailable, are "chafing at the bit." But active military service is largely a *young man's* game, and most of those who did their bit so magnificently in World War I, *when they were young men,* may now serve their nation best by guarding the home front.

Meanwhile, let the young physicians continue to come forward and fill the many positions in which their services are so sorely needed in the Army. These positions include not only professional assignments in hospitals and dispensaries, but also duties with tactical units. In time of combat each military unit the size of battalion or larger must be served by medical officers.

Field medical service, to be effective, requires adequate personnel to man the aid stations and unit dispensaries; to supervise evacuation of casualties; to establish and render professional service at collecting stations and clearing stations; to staff mobile surgical hospitals, evacuation hospitals, convalescent hospitals, field laboratories, and field medical supply installations.

Behind the combat zone in theaters of military operation, as well as in the zone of interior, medical men are needed at air fields, on hospital trains, at station hospitals, general hospitals, convalescent camps, dispensaries, laboratories, medical supply depots, and training centers; and to form medical personnel replacement pools, as well as for service with medical detachments assigned to tactical combat units.

The wide dispersion of our Army throughout the world requires proportionately more medical personnel than would be needed if our troops were concentrated in one principal theater of military operations, such as occurred in World War I. It is the responsibility of American medicine to see that these necessary personnel are made available.

The role of contemporary medicine in the current war effort is, indeed, a role of utmost importance which carries a responsibility of great magnitude. It calls for the maintenance of the health of civilian populations, especially industrial workers, and it demands adequate care for the health of our Army and Navy at home and abroad, for this is a war of annihilation or survival. Without conservation of our fighting strength we cannot hope to preserve civilization.

THE ARMY'S MEDICAL FIELD SERVICE SCHOOL AND ITS WAR TRAINING OF MEDICAL OFFICERS

COLONEL EDGAR ERSKINE HUME, MEDICAL CORPS, U. S. ARMY
CARLISLE BARRACKS, PA.

THE most extensive and most important work in the training of American medical officers for field duty in the present war is being done at the Medical Field Service School at Carlisle Barracks, Pa. At one of the Army's oldest and most historic posts there are being trained thousands of medical, dental, veterinary, sanitary, and medical administrative officers for the greatest war effort our country has ever had to put forth. Under the pressure of necessity, the efforts of a faculty of selected officer instructors are bent toward giving as much field training to as many officers as the military situation permits. We have many medical men in service. We need many more. Training goes on without cease, for there is ever the consciousness that no amount of money, no gifts of a generous and frightened Congress can buy us yesterdays. We have everything now needed to create a winning Army, save time. But even without as much time as we should like we can do great things with the fine officers and men who are flocking into the Army's Medical Department. We can overcome even the handicap incident to curtailed periods of instruction and turn out the men who will play the Medical Department's role in the winning of the victory.

WHY SHOULD AN ARMY HAVE MEDICAL OFFICERS AND MEDICAL SOLDIERS?

An unthinking person may feel that the only duty of a medical officer of the Army is to treat the sick or wounded man when brought to him for that purpose. Were this true, the Army would need no medical service at all. It would merely be necessary to employ civilian physicians and surgeons from time to time whenever and wherever needed. It is to be feared that some medical men otuside the military service are of this opinion.

But, unfortunately, it is not so simple. The Medical Department has many functions, all of which grow out of its great task *To Conserve Fighting Strength*—words which constitute the motto of the Medical Field Service School. It is our duty to select for the military service only those men who are physically fit for the duties expected of them, and thereupon, having admitted them, to keep them fit. Preventive medicine, in other words, is more valuable to the Army than is curative medicine, important though the latter is. Wars are won by men and not merely by machines. No matter how highly developed become our aircraft, our tanks, our submarines, and the rest, it is the human factor in the end that wins or loses wars.

Morale is an intangible thing, but one of supreme significance to the soldier. It is like the temper in the Damascus blade. Soldiers with high morale win

through to victory. Soldiers of poor morale have lost the battle before it begins. This is especially true of the officers, for officers with low morale cannot inspire their men to do deeds of military valor. Everyone knows the old adage that an army of lambs led by a lion is better than an army of lions led by a lamb.

All this is by way of stressing the importance of the Medical Department in the maintenance of morale. Fighting men must feel that if they are wounded or ill they will receive adequate medical care. They are encouraged and heartened by the knowledge that the medical soldier and the medical officer will accompany them right into action. There is no place that the infantryman goes, for instance, that the medical soldier does not go. Officers of the line have a way of telling their men that when frightened—and all soldiers are frightened at times—it is a good idea to fire in the direction of the enemy. Somehow this renews courage. It is the good old American spirit of striking back. Even though the riflemen may know that the chance of his hitting one of the enemy is small, he is nonetheless cheered by his ability to strike a blow.

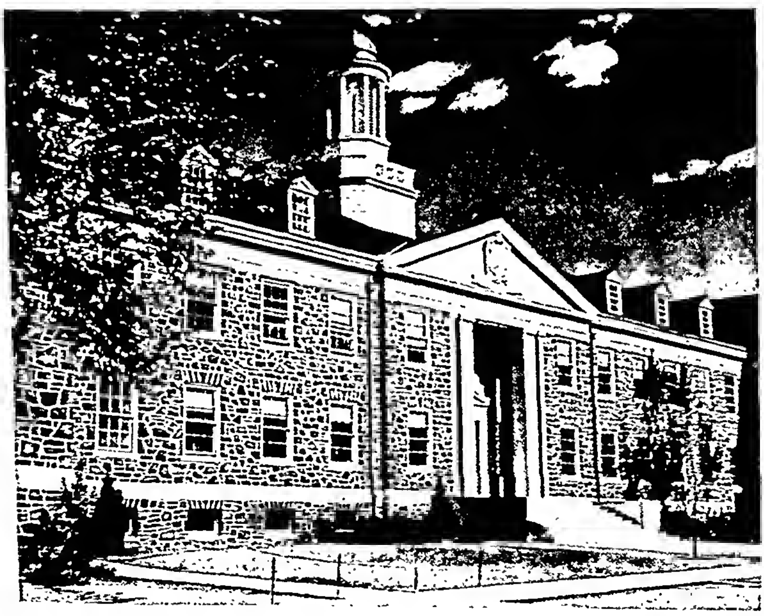


Fig. 1.—Hoff Hall, Medical Field Service School, Carlisle Barracks, Pa. Principal academic building. (Photograph by U. S. Army Signal Corps.)

But the medical soldier cannot do this. He is unarmed. His task is to care for the wounded men in action; he has no rifle with which to fire at the enemy. Only under rare conditions are medical soldiers armed; only, in fact, under the Geneva Convention, when it is necessary for them to defend wounded in their care. It takes a high order of courage for the unarmed man to stand up under the fire of the enemy and hold his ground. But that is exactly what our medical soldiers have always done.

Thus not merely from the humanitarian side is the Medical Department of the United States Army needed. As a morale builder it is a positive asset to the Commander-in-Chief. All this is taught the students at the Medical Field Serv-

ice School, but it is all predicated on our having thoroughly trained medical officers and men and military as well as medical training.

CARLISLE BARRACKS NOT A REPLACEMENT CENTER

The Medical Field Service School, Carlisle Barracks, is not a school for the training of medical soldiers; that is, enlisted men of the Army Medical Department. Its job is essentially one of training Medical Department officers, though a short course for selected noncommissioned officers is offered annually in time of peace. There are conducted, also, classes for enlisted Sanitary Technicians, of approximately one month each.

The task of Carlisle Barracks is "train the trainers." Many officers trained there are sent to the Replacement Centers where they assist in training the enlisted men. The Army maintains several such training centers: Camp Pickett, Virginia (formerly at Camp Lee, Virginia); Camp Grant, Illinois; Camp Barkeley, Texas; and Camp Joseph T. Robinson, Arkansas.

THE MEDICAL DEPARTMENT'S RECORD IN THE FIRST WORLD WAR

One may judge the future only by a knowledge of the past. We are engaged now in the most stupendous struggle that our land has ever known. Everything that we are and everything that we have is at stake. We at the Medical Field Service School are bending our every effort to impress upon the officers and officer candidates who take the various courses of instruction, the fundamental fact that they form an essential cog in the mighty military machine. They have before them the record of our service in other wars, particularly the first World War. Here are a few significant facts pertaining to that war:

> The first American wounded was a Captain of the Medical Corps; the second was an Army nurse. The first American killed was an enlisted man of the Medical Department. We have named one of our buildings Tugo Hall in his honor. The first American Army unit to reach Europe was a medical unit. The last to leave when we withdrew from the occupation of Germany was a medical unit. One may see the flags of these two units hanging side by side in the Army Medical Museum in Washington. Our battle death rates were higher than those of the Aviation, Cavalry, Engineers, Ordnance, or Quartermaster Corps. Those with a higher percentage than ours were the Infantry, the Tanks, the Artillery and the Signal Corps (the last but a fraction higher than the Medical Department).

These facts are eloquent. They show that while the Medical Department is what is sometimes called a "noncombatant service" (a misnomer for there is no such thing as a "noncombatant" in modern war), we, nevertheless, bear our proportionate share of danger. The missiles of the enemy do not discriminate, and the wearing of the red cross of the Geneva Convention is scant protection, as the foregoing data show. It must be remembered, too, that the soldiers with medical training go forward with the first elements of the advancing troops. And in withdrawals, some of which occurs in all wars, it is the medical unit that is the last to leave. Its duty in the place of danger is long.

MILITARY MEDICINE IS A SPECIALTY

Military medicine is a definite specialty, just as is ophthalmology or any other branch of medical science. It has its literature, its special practitioners,

and its ethics. It also has its handicaps, particularly one which does not affect any other medical specialty. This handicap is that in the past there has ever been a tendency for military medical officers to forget the lessons learned in the fierce light of warfare. Then, when once more war comes upon us—for there has always been a "next" war—it is necessary to learn military medicine anew, again in the hard way. The only practitioners of military medicine available have been the members of the small Medical Corps of the Regular Army and the medical officers of the National Guard and the Organized Reserves. These officers have been few, indeed, compared to the great number needed for war duty.

Fig. 2.—Bronze plaque in honor of Colonel Hoff. Placed within Hoff Hall, this tablet by the Gorham Company recalls a gallant medical officer's great contributions to training for field service.

DEVELOPMENT OF SCHOOLS OF THE ARMY MEDICAL DEPARTMENT

Nearly eighty years ago a wise Surgeon General, William Alexander Hammond (1828-1900), conceived the idea of forming an Army medical school in Washington, at which medical officers of our Army might receive preliminary instruction to fit them for their duties. He realized, as have all medicomilitary administrators, that the medical officer needs something in the way of training in addition to what he is taught at his medical school. Hammond went so far with his brain child as to select the professors, prepare the classrooms, and outline the curriculum. But his plans were disapproved by the Secretary of War. Mr. Stanton, who was his bitter personal enemy, finally had him tried

by court martial on a technical charge that resulted in his dismissal from the service. Years later Hammond was exonerated, but the Army Medical School had to await the advent of a more liberal Secretary of War.

Thirty years passed and the Surgeon General was the brilliant George Miller Sternberg (1838-1915), America's pioneer bacteriologist. Sternberg was more fortunate in the Secretary of War under whom he served, for Mr. Lamont approved of his plan to create the Army Medical School, so that in 1893 that institution came into being. It continues in its highly useful and protean work, filling a place that no other American institution occupies. The late Professor William Henry Welch (1850-1934) had a basis for calling it "America's oldest school of preventive medicine."

The Army Medical School has always given its students splendid training in certain techniques not ordinarily stressed, or considered only briefly, in the average medical school in the United States. Such work has included tropical medicine, helminthology, protozoology, advanced ophthalmology, and so forth. There used also to be found time to give a little practical instruction in drill, in equitation, and in field exercises. Such practical work was given in the grounds of the Walter Reed General Hospital or at Fort Myer. The Army Medical Center was not then contemplated, for the Army Medical School was miles away from the Walter Reed General Hospital, usually in rented quarters in downtown Washington. These arrangements were in effect until the end of the first World War.

In that great conflict medical officers once more learned military medicine, this time on a large scale. Contacts with medical officers of our allies, duty in far distant and hitherto unknown scenes, and highly specialized work, all had their broadening effect on American medical officers. When the war came to an end we had a highly efficient group of medicomilitary specialists in the Army. Then the huge army—at least it was huge by first World War standards—was demobilized. The Regular Army returned to its small size. The National Guard went back to the several States, and the Organized Reserves once more attempted to maintain a paper army and keep it trained with occasional periods of active duty in the summer, never more than a fortnight in extent. Had there been no other plan devised for keeping alive the lessons of military medicine bought at the cost of blood and human suffering, we would have been once more without any preparation when the second World War came upon us. But once more an able Surgeon General saw a way not only to keep alive what had been learned, but also to add to this sum of knowledge. That officer was Major-General Merritte Weber Ireland.

Surgeon General Ireland realized that thorough as was the training at the Army Medical School, that as essential as it is to the equipment of every medical officer of the United States Army, something more was needed. Fortunately for General Ireland's plan and fortunately for the Medical Department of the Army, there was already under the direction of the Surgeon General an ancient military post which was exactly what was needed. This was Carlisle Barracks, in the town of Carlisle, in the fertile Cumberland Valley of Pennsylvania, a hundred miles or so north of Washington. Carlisle Barracks was being used for General Hospital No. 31, devoted to reconstruction of disabled soldiers. It was

ideally located and all that was needed was for it to be turned over to the Surgeon General for permanent use as a training school for field duties of medical officers.

Accordingly, General Ireland addressed the following letter to the Secretary of War, through the Adjutant General of the Army:

"War Department
Office of the Surgeon General
Washington

April 28, 1920

"From: The Surgeon General.
"To: The Adjutant General.
"Subject: Medical Department Field School, Carlisle, Pa.

"1. I request that the U. S. Military Reservation at Carlisle, Pennsylvania, be permanently assigned to the Medical Department for use as a field school. Such a special service school is an urgent need in order that this department may comply with the policy announced in paragraphs 14, 15, 16, 29 and 30, G. O. 112, W. D., 1919.

"2. Facilities including construction exist at this post which may be converted to our needs in field training. Within two days by marching the prepared camp sites of the Gettysburg field are available. The use of adjacent mountain terrain for exercises has been offered to the Medical Department by a local townsman. The climate at Carlisle is such that alternation of season is found without undue extremes of heat and cold. The indoor season to be expected does not exceed three months.

"3. The need for the hospital at Carlisle will ultimately cease and the organization there will be listed for abandonment. If I understand correctly the use of this old post is not included in any General Staff plan for the future. I have considered, therefore, that the reservation could become available immediately and that there is no obstacle to its assignment to the Medical Department for field training purposes. Anticipating asking for a decision in this matter I have had made a preliminary survey of the site as to its suitability for this school. The results of this survey indicate that the selection will be an excellent one.

"4. It is my desire to have this school available about June 1 at which time a number of reserve officers and the present class of the Army Medical School would constitute the first class. In view of the urgency of this matter and the need of making arrangements for the abandonment of the hospital the earliest possible action is requested.

M. W. Ireland, Surgeon General, U. S. Army."

The Adjutant General of the Army, acting for the Secretary of War, indorsed the letter, under date of May 15, 1920:

"1. Upon the discontinuance of the United States General Hospital No. 31, the Medical Department is authorized to utilize for Medical Field School purposes the Carlisle Barracks Military Reservation, Penna., and the acreage adjacent thereto now occupied and controlled by the War Department by virture of permit from the Secretary of the Interior, dated August 22, 1918.

"2. The expenditure of funds by the Medical Department for the benefit of U. S. General Hospital No. 31, or the proposed Medical Field School, will be confined as closely as practicable to the territory contained within the boundaries of the Carlisle Barracks Military Reservation:

By order of the Secretary of War: G. H. Davis, Adjutant General."

Thus was born the Army's most important training center for medical officers in time of war. It has more than twenty-two years of efficient work to its credit and it has amply justified its inception. The post at which the Medical Field Service School was established is one of the oldest and most historic military reservations in our country. It merits a brief discussion of its history.

HISTORY OF CARLISLE BARRACKS

Carlisle Barracks was established in 1758 by the British Army, as shown by the "Military Papers" of Colonel John Stanwix, in the British Museum.

Stanwix was the first commanding officer of this post, established during the Seven Years' War, which was known in America as the French and Indian War. The British and Colonial troops occupied the present post during the long-continued hostilities with the Delaware Indians and other tribes. During the American Revolution, the Continental Army used Carlisle Barracks as a depot, and from there supplies and munitions were distributed. Hessian prisoners of war built the stone guardhouse at Carlisle Barracks; it is still standing and was until recently used for the purpose for which it was constructed. A School of Artillerists, probably the first school in the history of the United States Army, was established at Carlisle Barracks, 1778.

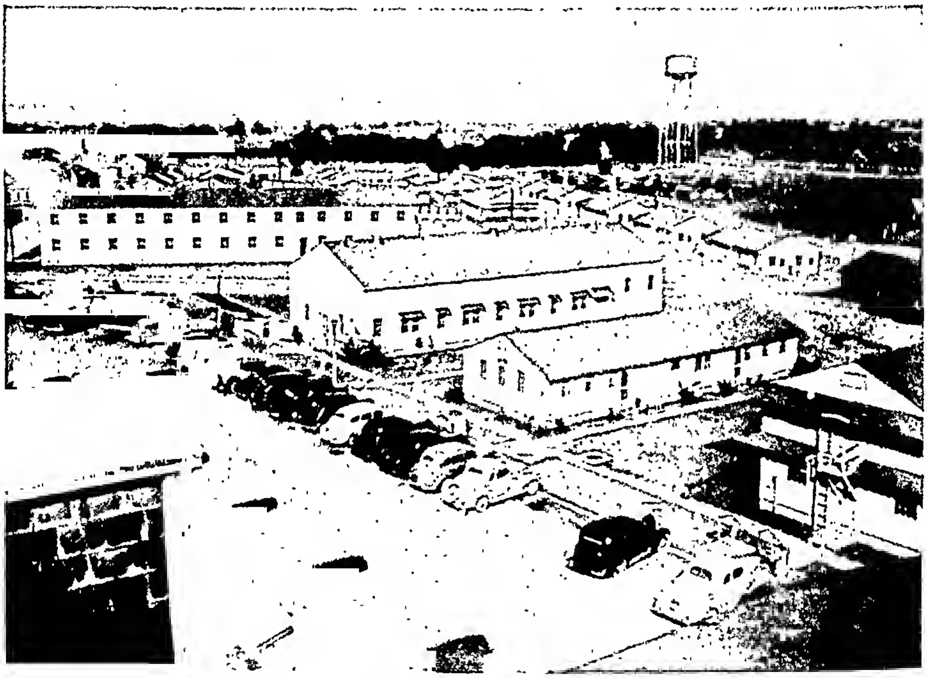


Fig. 3.—Temporary buildings at the Medical Field Service School. These are a few of the buildings for classrooms and barracks used for the accommodation of the large classes for officers and officer candidates at Carlisle Barracks, Pa.

American independence achieved, Carlisle Barracks was in the charge of the Ordnance Department of the Army, with the added function of being a recruit depot. During the Whisky Rebellion, in 1791 and 1792, President Washington sent an expedition, commanded by "Lighthorse" Harry Lee, father of General Robert E. Lee, against the four rebellious Pennsylvania counties. Carlisle Barracks was appointed the rendezvous for the Army. Some 10,000 to 15,000 troops were encamped on "the extensive common at Carlisle." President Washington came himself to Carlisle in the crisis of the rebellion, which was finally settled without bloodshed. At the time of the War of 1812 the old buildings used for the manufacture and storage of powder and shot during the Revolutionary War were reconditioned for the same purpose. When this second war with the mother country was at an end, a garrison, including both infantry and artillery, was continued at Carlisle Barracks.

In 1838 the War Department converted Carlisle Barracks into a recruit depot under the direction of an officer of dragoons. Here, in effect, was the Army's first cavalry school. At Carlisle Barracks, then, between 1838 and the outbreak of the Mexican War, were trained cavalrymen. From the post mounted units, both artillery and cavalry, departed for the southern theater of war. After that war the command at Carlisle Barracks devolved on Colonel Philip St. George Cooke, of the First Dragoons, a Virginian and General J. E. B. Stuart's father-in-law, who was destined to serve as a Union officer in the sixties. Shortly thereafter, a severe epidemic of cholera broke out, brought in, it was thought, by soldiers returning from Mexico. The post was quarantined, and the disease did not appear in the nearby town of Carlisle. Until the dark days of the War of 1861-1865 the post of Carlisle was typical of the United States Army station of the period, with the usual problems, vicissitudes, and development.

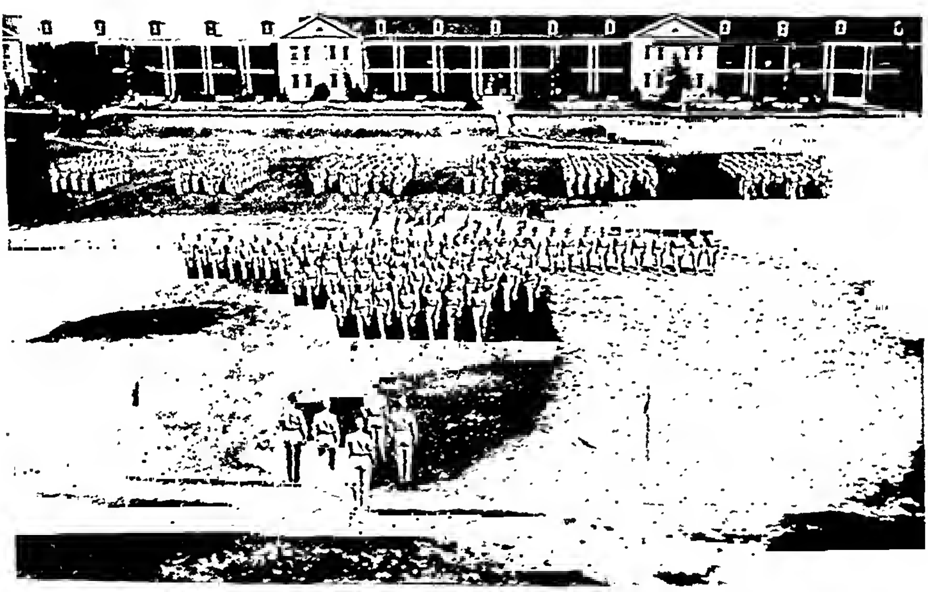


Fig. 4.—Parade of medical troops, Carlisle Barracks, Pa. Barracks of the 32nd Medical Battalion in background. (Photograph by James R. Gouffer of the *Evening Sentinel* of Carlisle.)

In the War Between the States, Carlisle Barracks became a supply center, as it had been during three previous wars. In 1861 it was the headquarters of the Mounted Recruiting Service of the United States Army. It not only saw the drilling of men recently taken into the service but also served as a place in which units withdrawn from the front were reconditioned, allowed to rest, and once more brought up to their strength. The fortunes of war were such that Carlisle Barracks was occupied by the Confederate troops, and General Fitzhugh Lee burned the buildings, with the exception, says a persistent tradition, of the quarters occupied by a West Point classmate. Thereafter, the post was rebuilt in part and surrounded by a wall within which drafted men were kept during their training. The war finally over, the post once more settled down to peacetime routine.

A part of the Sixth Cavalry was sent to Carlisle Barracks after the War. In 1867 it was suggested by the Surgeon General that selected enlisted men be trained as veterinary surgeons, and that the veterinary service not be placed under the Surgeon General, as the commanding officer of Carlisle Barracks had recommended. Thus a school for veterinarians was established at Carlisle Barracks. In 1871 the garrison of the post was ordered elsewhere, and for a time the post was closed, despite the pleas of the townsfolk of Carlisle. Matters dragged on until 1875, when Lieutenant Richard Henry Pratt (1840-1924), an officer deeply interested in the education of the Indian youth, established, under the authority of the War Department, the Carlisle Indian Industrial School. The Secretary of the Interior, Carl Schurz, gave it immediate support. Pratt, who ultimately rose to the rank of Brigadier-General, continued the direction of the Indian School until it closed in 1918. It made a splendid record during its nearly four decades of existence and achieved its objectives. The prowess of its students on the athletic field made it known to many who would not have been interested in the more serious side of its work.

In 1918 the War Department established General Hospital No. 31 at Carlisle Barracks, placing it under the command of Colonel (later Brigadier-General) Frank Royer Keefer, Medical Corps. The hospital did not actually begin to function until January, 1919, since much time was required for the conversion of the dilapidated buildings to hospital use. The institution became a reconstruction hospital wherein patients mutilated in the war not only were given adequate treatment but also were taught trades.

Before General Hospital No. 31 closed, Surgeon General Ireland requested the Secretary of War to assign Carlisle Barracks permanently to the Medical Department for use as a field school. This was done (see above) and the Medical Field Service School came into being on Sept. 1, 1920, the day that General Hospital No. 31 ceased to exist. The officer assigned as first Commandant was Colonel Percy Moreau Ashburn, Medical Corps. He was succeeded in 1923 by Major-General (then Lieutenant-Colonel) Charles Ransom Reynolds, who directed the school for eight years. His successors have been Brigadier-General Edward Lyman Munson (1931), Colonel Garfield Lesly McKinney (1932, acting), Brigadier-General Matthew Augustus DeLaney (1933), Brigadier-General Major Augustus Wroten Shockley (1935), Colonel Herbert Charles Gibner (1937), Brigadier-General Roger Brooke (1940), Brigadier-General John Mitchell Willis (1941, acting), and Brigadier-General Addison Dimmitt Davis, who has been Commandant since June, 1941.

THE FIRST FACULTY

The first faculty of the Medical Field Service School (1920) was composed of the following officers: Colonel Percy Moreau Ashburn, Commandant; Lieutenant-Colonel James Lung Bevans, Assistant Commandant; Captain Herbert Wellington Taylor, Secretary; Major Leon Connallin Garcia, Director of the Department of Administration; Captain Herbert Wellington Taylor, Director of the Department of Military Art; Major George Burgess Foster, Jr., Director of the Department of Field Sanitation; Major John Pierrepont Fletcher, Director

of the Department of Logistics and of the Medical Equipment Laboratory; Major Joseph Edward Bastion, Director of the Department of Enlisted Training and Commander of Demonstration Troops; and Captain Guy Blair Denit, in charge of Correspondence Courses. Many of these officers served at the Medical Field Service School for several years, and many of them have returned more than once for subsequent duty.

In 1923 Lieutenant-Colonel Charles Ransom Reynolds was appointed Commandant of the school, and to him is due its development and attainment of its important place in our scheme of military education. He served for eight years in this position of great responsibility, during which time he became known to nearly all medical officers of the National Guard and Reserves as well as those of the Regular Army. He became Surgeon General in 1935 and retired in 1939 to accept the directorship of the Bureau of Tuberculosis Control of the Commonwealth of Pennsylvania. Since General Reynold's administration of the Medical Field Service School no Commandant has served for as long as four years, due to the exigencies of the military service.

EXPANSION AND PROGRESS OF THE MEDICAL FIELD SERVICE SCHOOL

The Medical Field Service School, established as we have seen through the wisdom of the Surgeon General and the talents and hard work of its early faculty, did not spring fully developed into its present state. It was a gradual process, marked by many handicaps and delays. Instituted at the close of the first World War, it was given comparatively little financial support by the Congress of a war-weary nation. The most essential supplies were hard to get, and so short was the list of personnel that most of the officers had to occupy several positions simultaneously. It took good management, economy, and wise planning to keep it going. But those things it had, so that it passed through the hard times and was ready for the immense burden thrown upon it when the national emergency of 1940 arose. It was prepared for its present stupendous task of preparing the medical profession of the land for its part in the greatest war and the greatest danger that the United States has ever known.

Year after year the successive Surgeon Generals tried to obtain an appropriation for a new and adequate academic building at Carlisle Barracks. The school building first used was one of those built more than a half a century ago for the use of the Carlisle Indian Industrial School (1889). It was inadequate as to size, arrangement, and location. Finally, it was found to be actually unsafe, and the Secretary of War ordered it pulled down. Over the week end, for the matter was declared urgent, the old building was evacuated and the school was temporarily set up in the old gymnasium (1939). It was a matter of pride that not a day's instruction was lost in this wholesale change. But still no funds for a new building were forthcoming.

At length Congress in 1940 appropriated \$375,000 for a new academic building (Public 661, 76th Congress). Work on its construction began without delay and it was completed in 1941. The cornerstone was laid with appropriate ceremonies on June 26, 1941, by the Surgeon General, Major-General James Carre Magee. It was occupied by the Medical Field Service School on Oct. 6, 1941.

Thus when the emergency preliminary to the outbreak of the second World War was declared, the building was ready and has continued to serve as a nucleus of the school. With the enormous classes now being put through the school, it is, of course, inadequate in size, but with the temporary buildings constructed as classrooms there is sufficient space for the needs of the school.

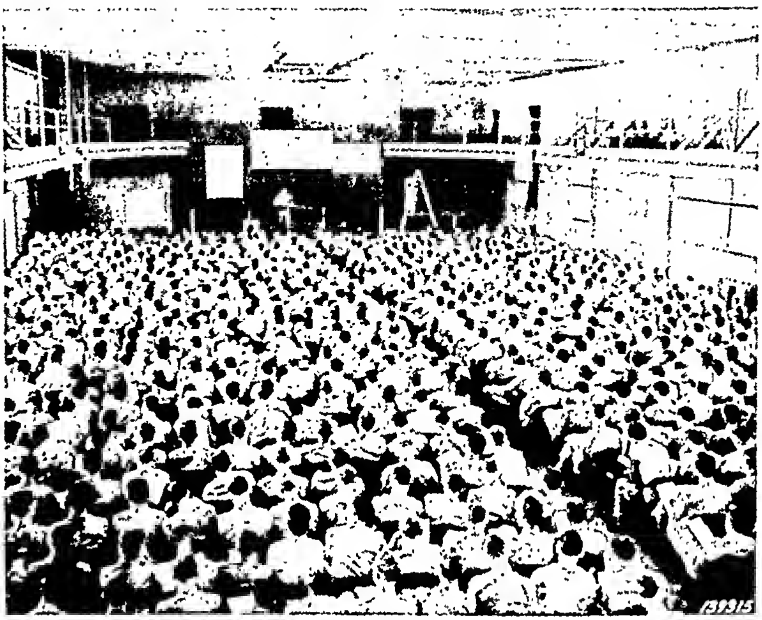


Fig. 5.—Officers class in medical tactics, Medical Field Service School, Carlisle Barracks, Pa. The school uses Tugo Hall, the gymnasium, for some of the large classes now under instruction. This building is named for Private Oscar C. Tugo of Boston, the first American soldier killed in the first World War. He was an enlisted man in the Medical Department. (Photograph by the U. S. Army Signal Corps.)

The academic building bears the name of the building that preceded it, Hoff Hall, named for Colonel John Van Rensselaer Hoff (1848-1920), pioneer proponent of adequate education in field duties for medical officers and enlisted men. On the cornerstone of Hoff Hall are carved words of a great British churchman, words that are as true now as when he wrote them nearly three centuries ago:

> "To Preserve a Man Alive in Midst
> of Chances and Hostilities is as
> Great a Miracle as to Create Him."
>
> *Jeremy Taylor.*

OBJECTS OF THE MEDICAL FIELD SERVICE SCHOOL

The objects of the Medical Field Service School are thus set forth in Army Regulations:

a. To instruct and train Medical Department officers of the Regular Army, National Guard, and of the Officers' Reserve Corps in the principles and methods of medical field service so as to increase their ability as instructors and to enhance their proficiency in the performance of their command and staff duties.

b. To instruct and train selected enlisted men of the Medical Department of the Regular Army, National Guard, and Organized Reserves in the duties of noncommissioned

officers pertaining to the organization, mobilization, training, and operation of Medical Department units of the components of the Army of the United States.

c. To act as an agency of the Surgeon General in the development and perfection of the principles and methods of medical field service.

d. To assist in the development of medical field sanitary equipment; to assist in the preparation and revision of Medical Department Training Regulations, instruction manuals, and other publications; to make research in matter pertaining to the field duties of the Medical Department; to disseminate to the service information pertaining to instruction and training used and developed at the Medical Field Service School.

It will be observed that these paragraphs from the "Army Bible" concern chiefly the Army as it exists in time of peace. Now that we are at war, the fundamental principles underlying the teaching at the Medical Field Service School have not changed, but the activities of the school have been largely directed in more specialized channels. There is no longer the division of the Army of the United States into the Regular Army, the National Guard, and the Organized Reserves, so that the instruction afforded at Carlisle Barracks is for all officers without distinction as to component of the large Army of the United States.

The teaching has naturally become much more elastic now than in days of peace. The curriculum has had to be radically changed several times, depending on the number of officers and others in each class, the period of time available for their instruction, the special duty to which the officer is destined by the War Department, the space available for teaching purposes and for quarters at Carlisle Barracks, and other factors. As information is received as to the medical service in our own Army and in those of other countries, the teaching at the Medical Field Service School is changed accordingly.

COURSES OF INSTRUCTION

As at all military schools, several courses of instruction are offered at the Medical Field Service School. Their nature and extent have been changed from time to time, depending on what military men call exigencies of the service. Prior to the declaration of the present emergency by the President, the following courses were being given at Carlisle Barracks:

Basic Course: This course was planned for junior or newly commissioned officers of the Medical Department of the Regular Army. It began early in January and normally was six months.

The National Guard and Reserve Officers' Course: Field officers (i.e., colonels, lieutenant-colonels, and majors) and senior captains of the Medical Department of the National Guard and the Officers' Reserve Corps attended for six weeks.

Advanced Course: Field officers of the Medical Department of the Regular Army, the National Guard, and the Officers' Reserve Corps received instruction for approximately three months.

Noncommissioned Officers' Course: This course, of approximately eight weeks' duration, beginning about September 15 each year, was provided for selected men of noncommissioned grades of the Regular Army, the National Guard, and the Organized Reserves.

Besides these formal courses at the Medical Field Service School, the three following types of summer camp were organized for instruction of officers and potential officers of the Medical Department of the Army:

Unit Training Camp for Reserve Officers: The officers receiving this instruction were, for the most part, assigned to medical units, though a few additional officers have been attached to the personnel of such a unit for the period of the camp. The units for which officers were so trained included medical regiments, medical squadrons, medical battalions, general hospitals, evacuation hospitals, surgical hospitals, and hospital centers. This camp has usually been of a fortnight's duration, beginning early in July of each year. Since the declaration of the existing limited emergency, this type of camp has not been conducted. Approximately 350 officers have been trained each year at this camp.

Basic Summer Training Camp for Reserve Officers: The instruction given in these annual camps, which also have been of two weeks' duration, has consisted mainly of basic military objects, including the employment of medical detachments and Medical Department units, Medical Department administration, mobilization, and sanitation. The chief objective of this camp has been to prepare the officers trained for more advanced instruction. Approximately 200 officers have enrolled in this camp annually.

Reserve Officers' Training Corps Camp: Here the course included instruction in the functions and activities of the Medical Department from the viewpoint of the enlisted man. The instruction included drill, function, and activities of medical detachments and of the medical battalion, squadron, and regiment, and general principles of sanitation, particularly in the field. The work at the R. O. T. C. camps supplemented that given by regular assigned instructors at various medical schools, and their instructors accompanied the students to the camp. The R. O. T. C. camps at Carlisle Barracks included 400 to 500 men each year and did not differ from similar medical R. O. T. C. camps elsewhere. At the camp held at Carlisle Barracks in 1941 the usual number of men were enrolled. The students successfully completing the full four years of Reserve Officers' Training Corps training were given reserve commissions in the Medical Corps of the United States Army.

Officers' Courses: The President's declaration of the existence of a limited emergency on Sept. 8, 1939, and of an unlimited emergency on May 27, 1941, changed the type of instruction offered at Carlisle Barracks, just as it did at other Army schools. There was no longer time to devote six months or more to a regular basic course; and the same was true of the other courses. A pressing need developed for training as large a number of officers of the Medical Department as could be and in as brief a time as possible. Therefore, a decision was made to establish courses of approximately one month each, known as *Refresher Courses.* The name was unfortunate, being borrowed from a type of course offered at some of the Army's other schools, designed for senior officers who needed a brief review. The new courses of Carlisle Barracks were attended largely by newly commissioned officers or those who had had little or no military experience, so that the courses were hardly "refreshers"; they were given to men who needed, and received, new information. This course is now known as the Officers' Course.

There were seven of these one month "Refresher" Courses given at the Medical Field Service School, beginning December, 1940. It was then decided that such brief courses did not permit sufficient training for the student officers, and so hardly justified their attendance. The period of training was, therefore, increased to two months, beginning with the course that opened on Sept. 2, 1941. Since then five two-month classes have been graduated, but the pressure of necessity then made it necessary to graduate five more of the one-month classes as well. Beginning in January, 1943, provisions for six-week courses will become effective.

Fig. 6.—Demonstration of work of medical soldiers in battle, Medical Field Service School. (Photograph by the U. S. Army Signal Corps.)

Officer Candidate Courses: Since the Medical Department of the Army has great need for officers to aid in its administration, but who need not hold medical, dental, or veterinary degrees, there was created the Medical Administrative Corps. Each Medical Administrative Corps officer releases a medical, dental, or veterinary officer for professional work, since the administrative functions would otherwise have to be performed by professionally trained officers. Any enlisted man of the Army who has had the minimum thirteen weeks' basic training may be recommended for admission to the Officer Candidate Class at the Medical Field Service School. Recommendations by the man's immediate commanding officer are based on his character, education, leadership, and other qualifications for commissioned rank. The Officer Candidate Course is three months in length. One group is admitted each month and one is graduated, so that a beginning, an intermediate, and a senior group are under training. A large proportion of the members of the Officer Candidate Classes are college graduates, and not a few hold professional degrees in law, engineering, or other nonmedical professions. Thirteen of these courses have been, or are being, held. This course in the future is to be given at the Medical Replacement Center at Camp Barkeley, Texas, will be related below.

Cadre Course: This course of one month's duration is designed to train medical officers being assigned to newly created divisions of the Army. Thought is given to the special needs of such officers, part of whose instruction is therefore different from that of the members of the regular officers' classes.

Medical Inspectors' Course: A one-month course for selected officers in field sanitation and preventive medicine. It is specially designed, as its name indicates, for officers who are to serve as medical inspectors, who correspond in duty somewhat to civil health officers.

DEPARTMENT OF THE MEDICAL FIELD SERVICE SCHOOL

The work of the Medical Field Service School is different from that of any other type of school that its students have known. It is so little understood by the public, that a consideration of the several departments is desirable. It will be observed that the school does not concern itself with the teaching of clinical medicine or with laboratory procedure. Such subjects are more properly taught at the Army Medical School and at its sister institution, the Walter Reed General Hospital, both at the Army Medical Center in Washington. The work of the Medical Field Service School and that of the Army Medical School is so arranged and coordinated by the Division of Training of the Surgeon General's Office that there is no conflict. There is likewise no conflict with the work of the School of Aviation Medicine at Randolph Field near San Antonio, Texas.

The officers in charge of the several departments of the Medical Field Service School have cooperated with me in preparing the following detailed statements, which have likewise been checked by the Assistant Commandant. The Assistant Commandant is in direct charge of the instruction given at the Medical Field Service School. His function is similar to that of a dean of a college or university.

Department of Military Art.—The mission of this department is the instruction of Medical Department personnel in the principles of medical support of combat operations. To this end, there are taught the basic principles of warfare, the military policy of the United States, the organization, tactics, and combat dispositions of the basic arm of the service, the Infantry, and the supporting arms and services, the Artillery, Cavalry, Armored Force, Air Corps, Engineers, etc. Correlation and mutual support of these arms and services in coordinated action are taught.

With combat tactics as a background, students are taught the principles of medical support of attack, defense, and special operations, such as river crossings, landing operations, retrograde movements, and pursuit. Early in the instruction students are taught the use of military symbols, map reading, and practical application of maps to terrain. Military maps include general, strategical, tactical, topographical, and photographic maps. Some of the exercises require close attention to orientation both in daylight and in darkness. Teaching methods include conferences, daily application terrain exercises, demonstrations, training films, map exercises, and examination.

Instruction is given in the preparation of medical and unit plans, preparation and use of standing operative procedure, and the formulation and issuance of combat orders to put plans into action. In addition to medical support of combat, instruction includes medical support of troops in bivouac and on the march.

The scope of the instruction in military art is such as to train medical officers in the principles of combat and the medical support thereof to such a degree that such officers can function efficiently under all conditions.

Department of Military Sanitation.—The old adage about the relative value of the ounce of prevention and the pound of cure is nowhere more true than in the military service. The Medical Department has the duty of treating sick and wounded soldiers. But it also has the far more important responsibility of keeping men well. To this end, all the teachings of preventive medicine and sanitation are used in the development of procedures designed for the maintenance of the soldier's health. In the field, in particular, modern sanitation is of the utmost importance. The course offered at the Medical Field Service School is designed to instruct students in the established practical measures for the preservation of the health of our fighting men. Thus, the prevention of disease is studied, not only as understood in our own climate, but in

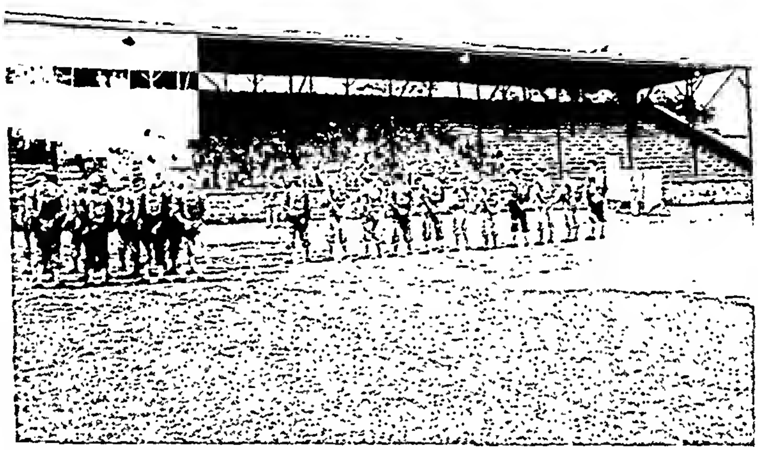


Fig. 7.—Demonstration of the work of a medical battalion. The instructor (at right) uses charts and diagrams. The medical soldiers actually demonstrate the principles taught. This exercise is on Stark Field, the only spot in America where troops have been drilled for every American War. (Photograph by the U. S. Army Signal Corps.)

the various other climates in which our forces may be sent by the exigencies of modern war in all its world theaters. Stress is placed on control measures against communicable diseases of military importance, including venereal diseases. Students are taught how to construct and maintain field devices and apparatus for the purification and safeguarding of water supplies, for the disposal of body and other waste, for the preservation, transportation, and preparation of food, etc.

The "Sanitary Area" consists of full-size models of various devices used by troops in the field, such as incinerators, water sterilizers, and the like. The control of insect and other vectors of disease is an important part of the curriculum. For instance, officers and officer candidates are taught how to protect the soldier from flies, lice, fleas, mites, ticks, ants, bedbugs, mosquitoes, midges, roaches, leeches, rodents, and a host of other pests and vermin. Of particular

importance is special consideration of jungle sanitation, the sanitation of commands in desert country, and in the cold climates of the far north. Protection against snakes and other venomous animals is studied. The proper methods of conducting sanitary inspections are taught.

At first thought one may suppose that military sanitation is no different from sanitation in civil communities, *mutatis mutandis*. But this is not true. In the Army the military situation must ever be supreme. The health of the men is to be maintained though the medical or health officer may have no voice in the determination of the nature of that situation. A civil health officer may place a placard on a house wherein there is a case of a communicable disease. Thereafter to enter such a house is to violate the law, and punishment can be brought about by the health officer. The military health officer, on the other hand, cannot say merely that certain area or place is dangerous because it contains communicable disease. He cannot order soldiers, in other words, to keep out of a dangerous area. He may well know that a certain place contains malarial mosquitoes, but if the military situation demands that the commander take his troops through that area, through it they must go. A military health officer must, therefore, be prepared to tell his general how best to prevent the occurrence of malaria, even though he knows that the men must be exposed. Our Department of Military Sanitation has not only to review the general principles and practices of sanitation, but also must teach its students how to make the best of situations actually to be faced. Actuality, and not theory, must govern.

Department of Administration.—Bookkeeping and the maintenance of records are boring to the average person. "Paper work," as it is termed by military men is, however, recognized as a necessity. No business firm or other establishment can run without records of what it is doing, and without the proper keeping of accounts and returns. Administration in the military service has been greatly simplified, but a minimum remains and always will remain.

The instruction of the Department of Administration, therefore, includes all the military activities of the Medical Department save the tactical and technical employment of units or personnel. This broad classification includes the methods of the procurement of supplies and the accounting for them. Supplies may be those acquired by the Medical Department (which like the Engineer, Signal Corps, Ordnance, and Quartermaster Departments, is a "supply department") or those used by the Army in general. Thus, students are taught how to make out the required reports, requisitions, returns, and other administrative procedures necessary in the proper care and disposition of sick and wounded soldiers. This includes the administrative procedures incidental to the procurement and maintenance of shelter and food for patients and medical personnel necessary to care for them. Military law is taught by this department, for all officers must understand court martial procedure, since such duty is occasionally unavoidable. The proper relations between military and civil power must be made clear. The organization and operation of the important military units of the Medical Department are taught, such as the various types of military hospitals.

In the instruction by this department, accuracy and rapidity of accomplishment of administrative duties are stressed. A considerable part of the time allotted to the Department of Administration consists of applicatory exercises in which the student is presented with actual or simulated administrative situations of the types likely to be encountered in actual service. These problems he is required to solve in accordance with prescribed Army administrative procedures.

Those who may think that it is a waste of time, in these days when time is so precious, for officers and officer candidates to have to learn administrative procedures, must remember the ultimate importance of adequate medicomilitary records. It is the Medical Department which must be prepared to tell the commander of troops just how many men he actually commands. It means little for a general to command a division, for instance, which has a strength of ten thousand men "on paper," if he does not know how many of his supposed strength are fit to fight. If 10 per cent of his command be sick or otherwise noneffective, then he commands not ten thousand men, but only nine thousand. He has only one source for the information that is vital to him and to the military success of his force. That source is the Medical Department. The senior medical officer can tell him—but only if proper records are kept by all medical personnel.

In our country we have always been generous in the matter of pensions, bonuses, compensations, and the like for former soldiers. In the old days when medical records were incomplete or wanting, many men could not prove their right to Governmental financial aid. Some worthy men, therefore, were not given the money to which they were entitled by law. In other instances, men were able to obtain money to which they were not entitled, because of incomplete records. Thus, the Medical Department of our Army has a double duty in the matter of keeping correct and adequate records. It must protect the rights of the future veteran, and it must protect the taxpayer. Records, then, are of very great importance.

Department of Training.—The training of soldiers is one of the prime functions of officers in any branch of the Army. Soldiers are made not born. No man springs fully armed from the brow of the Goddess of Wisdom nor from the brow of the God of War. Yet he must have some of the knowledge of Minerva, the skill of Mars, together with the cunning of Aesculapius, if he is to be a good medical soldier. The Department of Training devotes its energies chiefly to teaching officers and officer candidates how to train the men in the ranks. Its courses are designed to familiarize students with the basic principles of military instruction and the manner in which such subjects may be imparted to enlisted men of the Medical Department. The curriculum includes lectures, conferences, and demonstrations, all used to orient the student as to the general and special conduct of military instruction in its several phases. For purpose of physical training and body coordination, a systematic series of drill and calisthenic periods are conducted.

The work in military training is designed to familiarize the student with the methods of instruction useful in, and appropriate to, the military service. The mechanism of teaching is shown, with consideration of such factors as the

emotional and psychological approach, as required in training the medical soldier for combat duty. Particular emphasis is placed on the use of the visual sense for instructional purposes, and following this, by the applicatory method.

Military courtesy is taught both for itself and as an aid to achievement of military discipline. Instruction is given in both indoor and outdoor courtesies. Stress is placed on the relations necessary between officers and enlisted men, between officers, honors, and courtesies rendered to the flag, the National Anthem, commanding officers, and at ceremonies.

The inspection is a necessary feature of army life, since standards to be reached and maintained must at all times be closely scrutinized by officers concerned. The proper mode of conducting inspections is taught, including inspection of barracks and tents, unit inspections, and inspections of the soldiers' equipment. The class itself is periodically inspected by the commanding officer, which serves a direct purpose and likewise an indirect one, since it teaches how such duty should be performed.

In the preparation of training programs and schedules, the students are taught how to make the most of the time available for training their men, ever with an eye to the need for the particular unit being trained. From the estimate or analysis (the British say "appreciation") of the situation, a program for training is developed, out of which, in turn, a systematic schedule is produced. A part of the class instruction consists of an illustrative problem which takes them through the several procedures mentioned. Emphasis is placed on progressive step-by-step training, continuity of subject matter, variation in methods of instruction and approach, and decentralization of effort in teaching. Such schedules are worked out in detail in the classroom, and discussion is encouraged.

Instruction is given also in the movements of dismounted drill by demonstration and group instruction, followed by student application of each movement. To overcome awkwardness and diffidence, the class conducts cadence drill in which members collectively give the commands to the unit. This is followed by a period of command exercises in which each officer individually is called upon to drill the unit. Emphasis is placed on the disciplinary effect of drill, its possibilities in teaching precision and teamwork, and the instillation of prompt response.

By means of conferences, demonstrations, and application, the classes are instructed in litter drill, ambulance loading, physical training, use of the Army leg splint, and the assembly and adjustment of the medical soldier's field equipment.

Department of Logistics.—Logistics is defined as "that branch of military science which takes cognizance of the comparative warlike resources and capabilities of countries between which war is likely to arise, as well as of all conditions it is likely to be conducted, such as the geographical features, climate, means of transit, food resources, etc., of a probable seat of war." The more narrow application of the term logistics implies the study of the movement of troops and supplies, with reference to the factors which affect such movements.

At the Medical Field Service School the student is taught how to operate, maintain, and employ the motor transportation assigned to medical detachments, battalions, squadrons, and regiments. The teaching also includes study in-

Fig. 8.—Demonstrations of the use of the wheeled litter. (Photograph by the U. S. Army Signal Corps.)

Fig. 9.—An operating table made by placing ends of the litter on two medical chests at the Aid Station. (Photograph by the U. S. Army Signal Corps.)

volving the intricate problems of supply of medical units, and the problems incident to the medical supply of all units in the field.

The matter of transporting medical personnel and medical material from one place to another is far from being as simple as it may appear at first thought. Without a knowledge of the broad problem, one might think that when one wishes to move medical units from point A to point B, one has merely to look on the map and find the nearest road connecting the two points and order the medical units to proceed over that road. But perhaps that obvious road may be under the enemy's fire; or perhaps it is being used by our own advancing infantry; or perhaps we have just learned that the bridges over which that road passes have been destroyed. Thus one must be prepared to find other roads or to move across country in some instances. It all involves not only a knowledge of the terrain, but up-to-the-minute knowledge of the military situation, where our own troops and those of the enemy are disposed, and full information as to the condition of our vehicles and of the roads. And many other factors besides. Logistics is not a simple subject.

Department of Field Medicine and Surgery.—A relatively new addition to the Medical Field Service School, having been created in June, 1941, is the Department of Field Medicine and Surgery. The department collects and evaluates current information as to medical service in the field, particularly during combat. This information is communicated to the students and other personnel of the school. New drugs, new types of treatment, and new apparatus are studied. Matters pertaining to the prevention and treatment of trauma and disease in forward battle areas—the divisional area in general—are included.

This department teaches officers of the Medical Department how to make the best use of the material available at a certain place and under certain conditions. The Medical Department rarely, if ever, has the choice as to such conditions. Officers must learn to carry on with what they have; they should not be helpless if they are not provided with every convenience to which they may have become accustomed in civil practice. In these days of modern hospitals with fully-equipped clinical aids, some of the resourcefulness and ingenuity of the older generation of practitioners has been lost. Such qualities are essential in the military service. On the field of battle the medical officer must be prepared to treat wounded men with the best emergency measures available. Treat them he must, whether or not his supplies have arrived, or whether or not they have been exhausted.

This department considers such matters as first aid at the site where the injury has been sustained; emergency medical and surgical treatment in the battalion, collecting and clearing stations; principles and practices in the use of plasma, human albumin, and human blood, wherever applicable; prevention and treatment of shock, hemorrhage, and infection; principles and practice of the triage or sorting of the wounded; chemotherapy in its application to field medicine; medical care of gas casualties; use of anesthetics in the field; use of personal protective equipment and devices in relation to the prevention of injury and disease; the physiology of wound healing and the emergency management of wounds; consideration of medical and surgical equipment, including sutures, ligatures, dressings, splints, medicaments, etc. The department sums up its

duties in the words: "The application of the best medical and surgical care of the greatest number of sick and injured soldiers at the front, with a minimum of trained personnel and material."

Department of Dental Field Service.—The prime duty of this department is the instruction of officers of the Dental Corps in the organization and functions of the Medical Department units, in order that such officers may accomplish their professional and administrative duties most effectively in both fixed instillations and tactical commands. The course emphasizes the cooperation of medical and dental officers, and prepares the latter to serve as auxiliary medical officers when circumstances require.

The officers' classes at the Medical Field Service School include officers of each of the several corps of the Medical Department. Much of the instruction is given to the entire class. However, there are certain administrative functions of medical officers, for example, which do not apply to the Dental Corps, and vice versa. While the medical officers are being given such instruction, the dental officers are given, in separate classes, the work which applies to them alone. This arrangement has been found most satisfactory to all concerned.

Some of the special instruction afforded dental officers includes: duties of dental officers of all units, *Army Regulations* of particular interest to the Dental Corps, dental examinations and surveys, training of dental assistants, dental field equipment and treatment of jaw fractures in the field (with approved methods of splinting). The instruction in these various subjects is by conferences, applicatory exercises, and demonstrations.

Department of Veterinary Field Service.—This department furnishes, particularly to officers of the Veterinary Corps, essential information concerning the prevention of transmission to military personnel of the communicable diseases of animals. Thus special study is made of the protection of human beings from unsound food of animal origin (milk, meat, butter, eggs, cheese, etc.). Veterinary officers are given special instruction in medical field service, special emphasis being placed on the hospitalization and care of animals.

The special training afforded dental officers in their particular duties during periods when medical officers are receiving training not applicable to the Dental Corps, holds true also of the veterinary officers. While the medical and dental officers are each receiving special instruction not of interest to the other group, the veterinary officers are under the instruction of officers of the Department of Veterinary Field Service for special training necessary for their duties.

Among the special subjects taught by this department are: the preparation and rendition of the field veterinary reports and returns, together with other administrative work concerning animals; and the supply and inspection of food products. Other topics are the preservation of animal health, methods of preserving and storing foods of animal origin, veterinary aspects of chemical warfare, technical and tactical employment of veterinary units. Special attention is paid to the veterinary facilities with various arms and services. This last includes the care and treatment of animals under field conditions.

General Instruction.—Besides the teaching in the departments just outlined, special instruction is afforded in a number of special fields not directly connected with any department. There are lectures and conferences on such

topics as public speaking, consideration of medicomilitary problems of the past and present, notes on the medical services of the United States Navy, the United States Public Health Service, the Veterans' Administration, and that of various foreign armies.

Gettysburg is only about 28 miles from Carlisle Barracks, so that the classes, when time permits, are taken there for a tactical visit to the battlefield. There an officer explains the battle in detail, including the medical problems that arose. The battlefield of Gettysburg is used for map problems by every school in the United States Army, but only the Medical Field Service School is near enough to have teaching take place on the terrain itself.

Extension Courses.—The department of extension courses has the duty of preparing instructional material for use in the training of officers and enlisted men, particularly those of the Medical Department. The material prepared includes a lengthy series of Field Manuals, Technical Manuals, Training Films, Film Strips, and the Army Extension Courses. These training aids and texts include material on many phases of medicomilitary training. The data prepared by this department are distributed to the various posts and stations of the Army, and are utilized by officers and men in their training for war duties.

The series of correspondence courses formerly offered by this department is not being used under war conditions, since more extensive training is now permitted, and few persons not on active military duty would be interested in pursuing such courses.

Liaison Officers.—A modern army is a complicated machine. In order to understand the proper function of any of its parts, it is essential that the general working of other parts be thoroughly understood. It is like working a jigsaw puzzle. One must know the shape of the pieces already on the board before he can fit the piece in hand into the complicated mosaic of the whole.

To achieve this, the Medical Field Service School has several officers of other branches of the United States Army on its faculty. They teach the students the work of their particular branches and give information to other departments of the school on the technical and tactical side of their work. This system has been found most effective. There are medical officers, graduates of the Medical Field Service School, on duty at most of the other service schools of our Army. At the Medical Field Service School there are at present on duty as liaison officers, senior officers of Infantry, of the Armored Force, and of the Chemical Warfare Service. We are also highly fortunate in having a Colonel of the Royal Army Medical Corps of Great Britain, an officer of wide experience and training, who not only gives the greatest assistance in the teaching and the preparation of programs and plans, but also frequently visits medical installations in the field in many parts of the United States. He has been of great value in the work of the School. An officer from the Medical Field Service School has visited the Royal Canadian Army Medical Corps Training Centre at Camp Borden, Ontario, to study the work of that school and to give lectures on the work of the American school. Similarly, officers from the Canadian school have made visits to Carlisle Barracks for similar purpose. Such exchanges of visits will be continued.

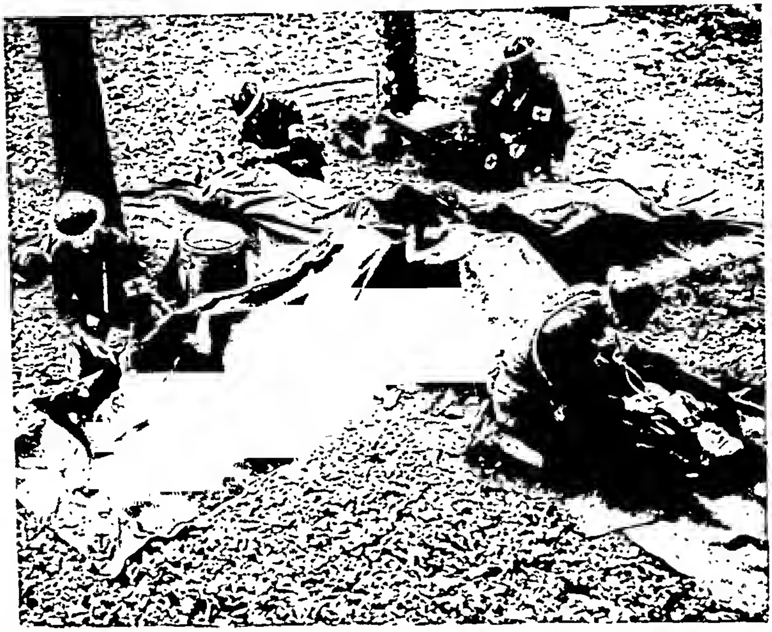


Fig. 10.—Work of the Aid Station. Patients kept warm while data are recorded and preliminary examinations made. (Photograph by the U. S. Army Signal Corps.)

Fig. 11.—Work of a Battalion Aid Station. Demonstration at Carlisle Barracks. (Photograph by the U. S. Army Signal Corps)

Contact with other branches of the Army and with the Medical Corps of the Navy and of the United States Public Health Service is readily maintained by means of visits of personnel to Washington, which is only a hundred miles distant.

PUBLICATIONS

The Medical Field Service School publishes three important scientific periodicals. They are all quarterly publications and they are all edited in the Surgeon General's Office, War Department. They are *The Army Medical Bulletin* (established 1921 and now in its sixty-fourth issue), *The Dental Bulletin* (now in its thirteenth volume), and *The Veterinary Bulletin* (now in its sixteenth volume). From time to time special texts are published. Such books are primarily for use in the instruction of the various classes at the Medical Field Service School. Other texts of wider application are used throughout the Army, and are often adopted as texts by schools and colleges not connected with the miltary service. Most recent of the latter class of books is *Elements of Military Hygiene*, the second edition of which is just off the press. It was written primarily for Cadets at the United States Military Academy, but it will doubtless have other general use.

Some of the material is issued in mimeographed form, but the greater part is printed. The Print Shop of the Medical Field Service School is now working twenty-four hours a day and is turning out an immense volume of material. The *Army Medical Bulletin* has a circulation of nearly 20,000; the *Dental* and *Veterinary Bulletins* have somewhat less.

The Book Shop of the Medical Field Service School sells the publications for practically cost to persons not students. Students are issued all necessary texts without cost.

The Art Department of the School prepares military maps, sketches, charts, diagrams, overlays, and the like, for use in connection with instruction. Such material is often issued in large quantity.

NUMBER OF GRADUATES OF THE MEDICAL FIELD SERVICE SCHOOL*

COURSE	DATE COURSE FIRST GIVEN	NUMBER OF GRADUATES
Basic Course	1920	1,369
Advanced Course	1921	217
Field Officers' Course	1923	273
Officers' Course (formerly Refresher Course)	1940	6,838
Special Cadre Class	1942	376
Noncommissioned Officers' Course	1924	1,129
Sanitary Specialists' Course	1940	712
Officer Candidate Class	1941	2,132
Medical Inspectors' Course		51
	Total	13,097

SUMMER TRAINING CAMP	DATE CAMP FIRST HELD	NO. MEN COMPLETING TRAINING
Basic Training (June)	1925	1,620
Regimental Training (July)	1923	4,655
Hospital Commandus	1928 (discontinued in 1931)	188
Reserve Officers' Training Corps		6,198
	Total	12,661
	Grand Total	25,758

*As of December 1, 1942.

DEMONSTRATION TROOPS

It is obviously impossible to give practical instruction in medical field service without demonstration troops. Our wise Chinese friends tell us that one picture is worth a thousand words. It is certainly true in teaching military science and art. When the Medical Field Service School desires to show how military medical units are set up and how they function in the field, practical demonstrations are arranged. Actual aid stations, collecting stations, clearing stations, field hospitals, and the like, are set up before the eyes of the students. Students help in such demonstrations and the picture is indelibly impressed on their memories.

At Carlisle Barracks there are a series of what the soldiers call "wars"; that is, demonstrations of the work of the Medical Department in selected typical military situations. A simulated "front line" is fixed, and men taking the part of wounded are evacuated therefrom through the various stages, through the various echelons, to the rear where they are to receive treatment in the appropriate hospital. Of course, in such demonstrations the distances between units and stations is foreshortened. Otherwise the stage is set as nearly as possible like the real thing.

The First Medical Regiment, a part of the First Division, was long stationed at Carlisle Barracks and served as demonstration troops. When the reorganization of the army took place in 1940, and the large or "square"* division of the first World War was abandoned, it was necessary to change the demonstration troops at Carlisle Barracks. The First Medical Regiment was transferred to Fort Ord, California, since for a time the National Guard retained the "square" divisions. In its place at Carlisle Barracks there was created a new medical organization, the Thirty-Second Medical Battalion, some of the seasoned men of the First Medical Regiment being retained as a nucleus of the new body. The new or "triangular"* division of our present Army is much smaller than the old "square" division, and has a medical battalion instead of a medical regiment. Our new demonstration organization has taken over the splendid barracks constructed for the medical regiment and the work goes on.

The mission of the Thirty-Second Medical Battalion is thus to demonstrate to students of the Medical Field Service School the current methods of performing those military functions which are outlined in theory from the lecture and conference platform. It shows how the provisions of *Army Regulations* and the numerous *Training Manuals* should be carried out. Thus, its work differs somewhat from that of any other medical battalion, for it merely shows how to give medical support to an infantry division, but does not have actual care of patients. It could at any time take the field and perform such duties.

The Thirty-Second Medical Battalion has to be able to demonstrate the work of any medical battalion, and something more. It must also be prepared to demonstrate the work of other medical units, such as those which normally function in the front and in the rear of the medical battalion in combat. In

*The "square" division was so called because it consisted of two brigades, each including two infantry regiments with supporting artillery and service troops. A "triangular" division consists of three infantry regiments with supporting artillery and service troops. The "square" division included some 22,000 men and was in vogue in World War I. The "triangular" division of some 15,500 men is the only infantry division now found in the United States Army.

other words, its personnel must understand not only their own work, but also the work of many other instillations. To insure better discipline its men use rifles in drills, it being understood that in actual field operations rifles would not be used. Medical soldiers are unarmed, except for a small number of automatic pistols, authorized by the Geneva Convention for self-defense and for the defense of patients in their care.

Since the demonstration troops at Carlisle must be able to stage mimic battles, the men of this organization must be able to play the roles of infantry, artillery, and other troops for demonstration purposes. They are like a stage army, but a stage army that can actually do in real life the things they demonstrate on an outdoor "stage" covering many square miles. The officers, many of whom are graduates of the Command and General Staff School at Fort Leavenworth, must understand the tactics and technique of the other arms and services.

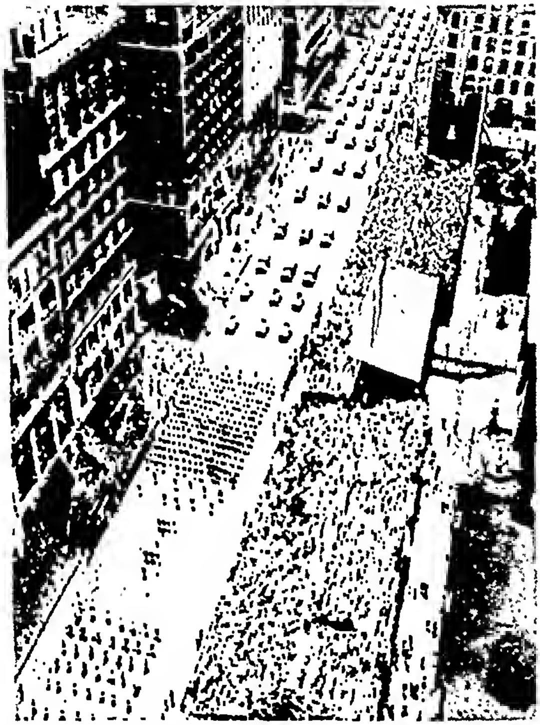


Fig. 12.—Carlisle Barracks Troops at the New York Flag Day Parade, June 14, 1942. This photograph by A. F. Sozio was taken without the knowledge of the 32nd Medical Battalion and the Medical Field Service School Band here seen passing the reviewing stand. The fine military appearance of these soldiers won them the official commendation of Lieutenant-General Hugh A. Drum, Commanding the Second Army.

The skill acquired by the personnel of the demonstration troops at Carlisle Barracks has resulted in frequent withdrawals of cadres of officers and men to form the nuclei of new organizations. They are the leaven which causes a new medicomilitary unit to develop to its desired size and skill.

EQUIPMENT LABORATORY

The Medical Department Equipment Laboratory is one of the important entities of Carlisle Barracks. Here are developed apparatus and appliances for use by the Medical Department in the field. New ambulances and other

vehicles are built and tested. New articles of equipment, such as first-aid packages and containers, field x-ray apparatus, litters, and equipment for battalion aid stations, are developed. Indeed, whenever a new problem as to medical supplies or equipment arises, it is this laboratory on which falls the duty of creating a model to fill the need. Many of the now standard articles of medical equipment were first only working models at the Medical Department Equipment Laboratory, and after passing rigid tests, were finally adopted by the War Department on the recommendation of the Surgeon General.

The variety of equipment and apparatus developed at the Medical Department Equipment Laboratory is very great. Some of the articles long in standard use in the military service had their origin in working models developed in this laboratory. Some of the more recent models include:

1. *Four-Wheel Drive Cross Country Ambulance:* With a capacity for four litter or seven sitting patients, this ambulance can operate over roads in any condition or cross country.

2. *Multiple Motor Ambulance (Bus Type)*: This vehicle operates at rapid speed over paved highways and at moderate speeds over rough roads. It has a capacity for 20 sitting patients or 12 litter patients.

3. *Animal-Drawn Cavalry Ambulance:* Even in this age of mechanization our Army still has some horse cavalry and horse-drawn artillery. Obviously, to be of service to such units, ambulances must be able to go wherever the units themselves go. This ambulance answers such requirements. Its low center of gravity makes it hard to overturn and makes it most effective on rough terrain.

4. *Mobile Surgical Hospital:* This unit is actually a hospital, contained in fourteen vehicles. There are seven large vans, of the semitrailer or bus type, four of which are complete operating rooms, the fifth a sterilization room, the sixth an x-ray room, and a supply room and office. There are also two power trucks for supplying electric current, two cargo trucks, one of which is a kitchen; one Engineer Purification Unit truck, and two command cars.

5. *Mobile Army Medical Laboratory:* One large motor van is thoroughly equipped for laboratory work in the field.

6. *Hospital Trains:* This includes a twenty-ton box car, for use in the combat area. The hospital train includes 16 ward cars, two cars for Medical Department enlisted men, one car for officers and noncommissioned officers, one dressing and dispensary car which includes the kitchen, and one utility car. Besides these the number of ward cars varies according to the immediate needs, depending on the number of casualties. A hospital train unit car, built by the Pullman Company in accordance with the specifications of the Medical Department Equipment Laboratory, was sent to Carlisle Barracks for study. It is designed to furnish food and operating room facilities for a full trainload of patients in the Zone of the Interior. Attached would be a baggage car and a variable number of sleeping or chair cars.

7. *Arctic Medical Unit:* Full equipment is carried in a pack by the medical soldier on skis. In transporting the wounded the skis and litter form a sled for the patient while the medical soldier uses snow shoes.

Among the other pieces of equipment more recently developed are a portable field disinfector, wheeled litter carrier, pack carriers for equipment of medical detachments, folding arm splint, folding leg splint, flight service chest for use by medical units with the Air Forces, field operating light with generator, new individual equipment for the medical soldier and for the medical officer, a new first-aid packet, a portable still, chemical heat pads, neon emergency operating lights, and new medical equipment for parachute infantry. A recent development is the medical equipment for use with parachute troops, material that can withstand parachute dropping.

The foregoing articles of equipment were displayed by the officer in charge of the Medical Department Equipment Laboratory and his staff at the meeting of the American Medical Association in Atlantic City, June 6-12, 1942. The greatest interest was manifested by delegates and other visitors.

Medical Field Service School's First War Casualty.—On March 26, 1942, Major James Augustus McCloskey (1909-1942), Medical Corps, a graduate of the Medical Field Service School in 1937, was killed in action in the Bataan Peninsula, Philippine Islands. He is our first war casualty, though there have been others reported since. A tablet in his memory has been placed in the Chapel of the Medical Field Service School, dedicated with appropriate ceremonies.

Medical Administrative Officer Candidate School at Camp Barkley, Texas.—So great has been the need for officers for the Medical Administrative Corps that on April 15, 1942, the War Department ordered the creation of a second school for the training of approved candidates. It was established at Camp Barkeley, Texas, and was organized so rapidly that the first class began its instruction on May 9, 1942, and has since been graduated. This school is doing work comparable with that carried on at Carlisle Barracks, though, of course, with less equipment. No instruction of officers is given at Camp Barkeley.

While the Officer Candidate School at Camp Barkeley is not a branch of the Medical Field Service School, it is, in effect, an offspring of it. The Commanding General of the Medical Replacement Center at Camp Barkeley, Brigadier-General Roy Cleveland Heflebower, is a medical officer, and the Assistant Commandant and other members of the faculty of the Camp Barkeley School were taken from the faculty of the Medical Field Service School. The curriculum at Camp Barkeley is essentially the same as that of Carlisle Barracks. The medical soldiers at Camp Barkeley furnish ample demonstration units in connection with the training of the officer candidates.

Since the preparation of this report, the War Department has ordered that effective January 1, 1943, the Officer Candidate Classes at the Medical Field Service School be discontinued upon graduation. Thus by March 1, 1943, there will be no more student candidates under instruction. In future the training of candidates for commissions in the Medical Administrative Corps will be carried out at the Medical Replacement Center at Camp Barkeley, Texas. Thus the whole effort of the Medical Field Service School will be devoted to the training of officers. The course will be extended to six weeks instead of one month as at present. The special courses for officers will also be given at Carlisle Barracks as needed.

CONCLUSION

The medical profession of our country owes much to the Army Medical Department. Some of the greatest advances of American science have been made by our medical officers. Many of them are not well known as Army gifts. Army medical officers have *inter alia* given our country its first medical book, its first pharmacopoeia, its first textbook on bacteriology, its greatest bibliographical work—the Index Catalogue of the Army Medical Library. Army medical officers introduced vaccination to America, developed our present system of typhoid fever prophylaxis, showed that the mosquito transmits yellow fever, created America's first school of preventive medicine, developed the largest medical library the world has ever known, established the largest medical museum in America, first showed the importance of proper ventilation, created the first large American hospital (the New York Hospital), introduced photomicrography, invented the purification of drinking water by liquid chlorine, and many another piece of scientific research and achievement which now benefit mankind. They have successfully fought epidemics of many kinds and in many quarters of the globe.

The school developed by the Army Medical Department at Carlisle Barracks is, we believe, proving itself one of the Army's worth-while gifts to the whole country. It is worthy of inclusion in the above roll. If by its teachings it can prepare medical officers more efficiently to care for soldiers in the field, to prevent disease and injuries wherever possible, and to cure them when they have been unavoidable, it will have accomplished a noble mission. When such mission is one that intimately concerns every household in the land, it is of supreme importance indeed. To the end that our men may be the better protected, the Medical Field Service School devotes its every energy and its every thought. Its personnel are mindful of the full purpose of the school's motto: "To Conserve Fighting Strength." These words are not merely worn on the badge of the school that forms a part of its personnel's uniform. They are deep set into the conscience of every man connected with this seat of learning on which so much now depends.

Fig. 13.—Coat of arms of the Medical Field Service School.

THE MANAGEMENT OF THORACIC WAR INJURIES*

James H. Forsee, M.D., Lawrence M. Shefts, M.D., Benjamin Burbank, M.D., Leo J. Fitzpatrick, M.D., and Thomas H. Burford, M.D., U. S. Army

THE management of thoracic injuries has always constituted a serious and significant problem. The frequency of chest injuries and the attendant high mortality demand more than passing attention. Berry,[4] in quoting Hoche's review of 11,000,000 wounded in World War I, stated that thoracic wounds were third in order of frequency and the mortality of this group was 56 per cent, whereas the total mortality was only 8 per cent. Further, in reviewing the cause of death among 12,350 killed, Hoche found that thoracic injuries accounted for 20 per cent and was second only to head injuries. DeBakey,[14] in his excellent collective review, quotes Sauerbruch, Crafoord, Trueta, and Loeffler as finding the frequency to be from 12 to 40 per cent. Such frequency and mortality justify a serious and detailed consideration.

That a considerable proportion of deaths occur near the scene of the trauma is evidenced by Duval's statistics, as quoted by DeBakey.[14] He found that the mortality decreased progressively from advanced aid stations to base hospitals, being 30 per cent at aid stations and 15 per cent in base hospitals. The one factor which can and will reduce such fatalities is the earlier definitive treatment by surgeons trained in the management of thoracic injuries. To effect this it is necessary, in the mobile war now occurring, to have an equally mobile system of evacuation and treatment, flexible enough to meet casualties soon after the injuries are incurred. This entails first, the special training of surgeons and their assistants in the care of chest wounds, and second, an organization whereby they are available on short notice to function in properly equipped medical installations at the point of maximum casualties close to the battle front.

The success of such an attempt in reduction of mortality depends largely on the ability of the personnel and the proper functioning of the medical installations in which they work. Surgeons especially trained in thoracic surgery not only must be used in carrying out the necessary surgery but also as instructors in training general surgeons in the immediate care of thoracic injuries. There are too few specialists in thoracic surgery of field age available. This deficiency can be remedied by the training of general surgeons in the fundamentals of thoracic physiology. This training could best be carried out in hospitals where chest cases would be concentrated. Such centers would also permit training in bronchoscopy and would make possible the training of anesthetists in the technique of endotracheal anesthesia. We feel that as the advent of endotracheal positive pressure anesthesia has been a very important factor in the great advances of thoracic surgery in the last decade, it can be an equally important

*From the Second Auxiliary Surgical Group, Lawson General Hospital, Atlanta, Ga.

factor in the decrease of mortality in war wounds of the chest. Such a program would fulfill the necessity for early definitive treatment by trained personnel.

The second factor involved is one of time and distance. Reduction of the interval of time between injury and definitive treatment resolves itself into either having the surgeon immediately available to the fast changing mile-devouring fronts or having rapid evacuation of the wounded by airplane or motor to the surgeon.

A program of early definitive treatment by trained personnel as outlined above will not only reduce mortality of thoracic injuries but also decrease morbidity, reduce the number of days of hospitalization, and eliminate a large number of chronic empyema cases which linger on long after the war is over and which are the obvious result of treatment usually applied late and incorrectly. The elimination of such a large group, requiring hospital care for many years, would be economically sound.

The more important problems concerned in a presentation of the management of thoracic injuries include a discussion of the relation of wounds to types of projectile, shock, hemorrhage, open pneumothorax, tension pneumothorax, interstitial pulmonary emphysema, massive collapse of the lung and contrecoup injury, "blast lung," ecchymotic mask, cardiac concussion, wounds of the heart and pericardium, cardiac resuscitation, acute and chronic empyema, foreign bodies, combined abdominothoracic wounds, chemotherapy, and anesthesia in thoracic injuries.

RELATION OF TYPE OF PROJECTILE TO WOUND

The steel-jacketed small caliber bullet, as used in small arms fire, will result in a perforating wound. Although the path of the bullet from point of entrance to point of exit may be very bizarre, ricocheting far from its original line of entry, the wound caused is not of the type produced by high explosive. Its long and narrow path is marked by a coagulated and seared trail. However, in injuries from shrapnel, high explosive artillery shells, hand grenades, bombs, or debris of falling buildings, large lacerated wounds of the chest result with serious prognosis.

In receiving casualties from a given sector, the knowledge of the type of small arms used and the type of high-explosive shells laid down by the artillery can usually be ascertained; this knowledge is important in planning the care of casualties already present as well as preparing for the expected additional wounded.

SHOCK

When a patient is first seen with a chest injury, it is often difficult to determine whether the shock present is due to hemorrhage or to a disturbance in the cardiorespiratory mechanism. In such cases any obvious source of hemorrhage must be immediately controlled, and heat and oxygen therapy must be promptly administered. Such conditions as sucking wounds of the thorax or rapidly progressing tension pneumothorax will produce the symptoms of marked shock. Numerous fractured ribs approaching the "stove-in chest" or "hinge fracture" with its many anterior and posterior fractured ribs should be immediately immobilized. The correction of the abnormal mobility of such a chest will

rapidly decrease the intensity of the shock. The simplest and often the most efficient immobilization is the circumferential strapping of the lower costal margin, as advocated by Blades.[6] A rapidly decreasing vital capacity as the result of pulmonary collapse from bleeding into the pleural cavity can simulate the complete clinical picture of shock. The reduced vital capacity has as a result a proportionate anoxia. The resultant shock is generally not due solely to blood loss into the pleural cavity but to the sudden decrease of vital capacity associated with mediastinal shift. In such instances the patient should be placed in a semisitting position, since he is usually more comfortable and can breathe more easily in this position. The same position should be used following treatment for any condition wherein the vital capacity has been decreased. Vital capacity is often further decreased by reduced respiratory excursion resulting from pleural pain, the most common cause of which is fracture of the ribs.

The treatment of shock in chest injuries requires the same close attention as it does in other injuries producing shock. Plasma transfusions are especially valuable in correcting hemoconcentration wherein actual blood loss has not been marked. In chest injuries, however, immediate attention must be given to correcting the abnormal thoracic physiology. A sucking wound of the thorax must be closed, a tension pneumothorax must be relieved, and hemorrhage from intercostal or internal mammary vessels must be controlled. Morphine is indispensable for the relief of pain in combating or preventing shock. It is also important to remember that considerable loss of body heat occurs in open wounds of the chest and immediate closure is the only effective control.

HEMORRHAGE

Hemorrhage in thoracic injuries not involving the heart is from one of three sources; chest wall, parenchyma of the lung, or from the large vessels at the hilus of lung. A hemothorax may occasionally occur from contusion of the chest.

Severe hemorrhage from the chest wall arises from either the intercostal or the internal mammary vessels. When circumstances do not permit a careful exposure of a bleeding intercostal vessel, two circumcostal sutures including the rib, placed above and below the bleeding point, may suffice. In this connection the anatomic position of the intercostal vessel should be recalled. The intercostal artery medial to the angle of the rib is approximately in the center of the intercostal space, and from that point anteriorly the vessel progressively approaches the inferior border of the rib above so that from the angle of the rib forward the vessel is under the edge of the rib in the subcostal groove and simple circumcostal massive suture may be insufficient. In hemorrhage from the internal mammary artery it is imperative to ligate both ends of the lacerated vessel since hemorrhage can occur from either end. The vessel is located approximately one-half inch lateral to the edge of the sternum. Exsanguination may occur in a few hours from unchecked hemorrhage from the internal mammary artery. Hemothorax in which there is no lesion of the chest wall or parenchyma of the lung may occasionally be caused by contusion of the chest.

Hemorrhage due to injuries of the parenchyma of the lung will often be associated with a pneumothorax and a hemopneumothorax will result. In the absence of fractured ribs or wounds in the region of the intercostal or internal

mammary vessels it may be assumed that the etiology of a hemopneumothorax is parenchymal bleeding. Hemoptysis may or may not occur. The immediate effect of parenchymal bleeding into the pleural cavity will be a compression of the lung and the compression often stops the bleeding. The after treatment of hemothorax once the hemorrhage has stopped resolves itself into any method that will re-expand the collapsed lung. There are in the main three schools of thought: first, energetic aspiration repeated often enough to keep the chest dry; second, watchful waiting in which aspirations are performed only to relieve cardiorespiration embarrassment; third, a line of treatment that is somewhat midway between the two. This consists of aspirations (not primarily aimed at keeping the chest dry) and of partial replacement with air. The first has the advantage of removing an excellent culture media, but it has the disadvantage of possibly reviving the hemorrhage due to the high negative pressure resulting from removal of a large amount of blood without replacement with air. The second is advantageous only in that less exertion is necessary in treatment. It has the definite disadvantage of a long period of hospitalization awaiting absorption of blood and re-expansion of the lung with the probability of a resultant fibrothorax, and prolonged chronicity. Further, should infection develop it would occur in a large pleural space and a total empyema often results. The third method is repeated (after seventy-two hours) with partial replacement by air. The aspirations are so spaced as to empty the pleural cavity of blood gradually. The air replacement should be controlled by noting the intrapleural (pressure) readings as recorded on a pneumothorax apparatus. If such an apparatus is not available, air may be replaced in a proportion of 50 per cent of the fluid removed. This was popularized by Morelli[39] in World War I.

Severe lacerations of the parenchyma, especially those extending into the hilus and involving the large vessels, may necessitate lobectomy or even total pneumonectomy. These will most often be associated with large open wounds of the chest and will be available to actual inspection. If adequate facilities are available and the condition of the patient permits, individual ligation lobectomy, as described by Blades and Kent[7] should be done. If hemorrhage is massive and the condition of the patient does not permit a lengthy operation, then massive tourniquet ligation is indicated. It seems probable that lobectomy will be employed with greater frequency in the early management of severe pulmonary injuries, and if properly employed, will, it is believed, be highly successful.

OPEN PNEUMOTHORAX

Trauma to the chest resulting in open wounds exposing the lungs is not always fatal. The same may be said of bilateral open wounds. Graham[16] and Bell proved experimentally that a wound exposing the pleural cavity could be tolerated if the amount of air entering the wound was not greater than the difference between the tidal air and original vital capacity. Thus the important factors are the size of the wound and the original vital capacity. The lung on the injured side does not collapse to a point wherein it is nonfunctioning when it is exposed to atmospheric pressures even when the pleural cavity is free of adhesions. During the respiratory cycle the pressures exerted on the exposed lung are not always atmospheric. The thorax is a flexible moving container during

the respiratory cycle, and there may be even negative pressures in the exposed side during inspiration.[18] This permits a rush of air through the glottis in answer to the negative intrapulmonic pressure occasioned by the enlargement of the chest on inspiration. Thus, if difference of pressures permit of the entrance into trachea of amounts of air to answer the tidal air requirement, the patient will not die of asphyxia. There is also the factor of interference with function of the contralateral side. In the healthy person the mediastinum which is free of adhesions and is not stiffened by previous inflammatory involvement permits transmission of pressures through it so that almost identical pressures may be found in both pleural cavities. Thus the decrease in vital capacity from an open wound of one side of the chest arises from both lungs. This has the practical effect of a tolerance of larger wounds in persons with a stiffened mediastinum.

If the amount of air entering the open chest wound during each inspiration is large enough to interfere with intake of the tidal air requirement, asphyxia will occur. In such cases an interesting and vicious chain of events ensues immediately. There is an initial decrease in vital capacity. The exposed lung will move to the degree that the mediastinum shifts with each unopposed inspiration and expiration of the contralateral lung. This resultant mediastinal shifting increases in rapidity as anoxia from the initial reduction in vital capacity becomes more marked. This shifting is termed "mediastinal flutter." Such shifting will have a direct effect on the right side of the heart. Since the base of the heart is fixed as compared to the apex, it will permit considerable movement of the apex. This shifting on the base follows the rapid fluttering of the mediastinum, and kinking of the large vessels entering the right side of the heart will ensue. Anoxia further is increased by the phenomena of pendulum respiration. On each expiration the air from the contralateral lung with its predominant carbon dioxide content is only partially exhaled, and part goes over into the main and secondary bronchi of the collapsed side. On the next inspiration the air taken into the contralateral lung is composed of atmospheric air from the trachea and air with a high carbon dioxide content of the air reaching the contralateral lung. Thus the chain of events starts with a reduction in vital capacity which involves both lungs, and each step further decreases it. The shifting mediastinum interferes with the proper filling of the right side of the heart, the progressive anoxia of pendulum respiration ensues and must be treated as an extreme emergency. To break the chain of events closing the wound of the chest is the method of attack.

The immediate treatment of open wounds of the thorax is simplicity itself. Closure of the wound is imperative. This can be done by gauze pads and adhesive applied so as to make an airtight dressing. As soon as possible it is essential that the wound be closed surgically, for it is seldom that a dressing of such a wound remains airtight for very long. If adhesive and gauze is not available, large safety pins may be used to close the wound. At the time of the dressing any obvious foreign bodies should be removed from the pleural cavity, and all foreign bodies should be removed when the wound is later closed surgically.

The essential point is that the open pneumothorax must be converted into a closed pneumothorax. The test of the efficiency of the emergency closure is the absence of the sucking noise accompanying respiration.

If the parenchyma of the lung is lacerated, closure of the wound of the chest will convert the open pneumothorax into a progressive tension pneumothorax. This then poses a problem in field conditions, for once the wound has been closed it is necessary to provide an outlet from the pleural cavity which is equal to, or greater than, the opening of the lung into the pleural cavity. This outlet must be a one-way valve, permitting the escape of air from the pleural cavity to the outside. This is accomplished by closure of the wound and installation of a catheter into the pleural cavity and connection of the catheter to a water-seal bottle. The water-seal bottle should always be kept below the level of the chest. No complicated apparatus is necessary, for under local anesthesia through a skin incision the catheter may be introduced into the pleural cavity through an intercostal space. If the patient is to be transported a considerable distance, the water-seal bottle may be too cumbersome and a rubber flutter valve may be substituted. Such a flutter valve may be made by slitting the finger of a rubber glove so that a flap is produced. If it is feasible to close the wound surgically shortly after it occurs, and if the lung is lacerated to a degree that simple suture is insufficient and the patient's condition precludes lobectomy, the lacerated portion of the lung may be sutured into the wound. This may tide a patient over a critical period and more definitive surgery may be done later under more favorable circumstances.

It will bear repetition that treatment of the accompanying shock, position of patient, oxygen, warmth, plasma transfusion, and sedation is essential.

TENSION PNEUMOTHORAX

Tension or pressure pneumothorax is the result of a pleuropulmonary connection.* This connection is the result of rupture of the parenchyma, and the tear acts as a one-way valve which permits the entrance of air from the parenchyma into the pleural cavity. There is no opening for the air to escape through an intact chest wall and the pressure in the pleural cavity will rise progressively. The rapidly progressing tension pneumothorax causes marked dyspnea and all accessory muscles of respiration are utilized. Cyanosis becomes prominent as the mediastinal shift causes interference with heart action and the veins of the neck become prominent. Dyspnea and further reduction in vital capacity result from mediastinal displacement. The displacement is away from the involved side. The patient sits in a semierect position, and as the dyspnea increases, the patient's worried anxious expression is as expected of one fighting for his breath. It is essential to decompress the pleural space immediately. Whether it is necessary to maintain continuous decompression depends on the size of the pleuropulmonary opening. Repeated decompression by needle aspiration may suffice, but if aspirations have to be repeated for more than once daily, it is probably far safer to install a continuous decompression. In injuries occurring on the battlefield the average case has a greater margin of safety by this method. This may be done by introducing a large bore, Nos. 14 to 16 gauge

*A tension pneumothorax may also result from a pleurocutaneous wound.

needle, into the pleural cavity, strapping it in place, being careful not to obstruct the lumen of the needle. If such needles are not large enough to accomplish decompression, an intercostal catheter connected with an under water-seal bottle must be used. Besides the patient's obvious dyspnea and cyanosis, a constant check on the efficiency of the decompression is the position of the trachea as palpated in the neck. As decompression is accomplished, the trachea resumes its midline position from its displacement away from the side of the tension pneumothorax.

INTERSTITIAL PULMONARY EMPHYSEMA

Spasm of the glottis is a reflex mechanism of sudden compression injury to the chest. As a result, air is trapped in the lungs and compression of the chest raises the intrapulmonic pressure suddenly, and rupture of alveoli in the parenchyma of the lung occurs. The pumping action of respiration forces air out into the interstices of the parenchyma. The air travels along the branches of the bronchi and blood vessels, ascending to the hilus and thence up the mediastinum.[14] The appearance of subcutaneous emphysemas at the base of the neck may be the first indication that such a condition exists. Roentgenograms will reveal presence of air in the mediastinum. This condition may be found in absence of any pneumothorax, fractured rib, or hemothorax. This condition has been noted in patients suffering from severe asthmatic attacks.

Treatment consists of watchful waiting. In most instances spontaneous recovery results. It is essential to note whether or not there is extension of the emphysema from the base of the neck outward to the shoulders, up into the neck, and down onto the chest. If this extension is present, then it is evident that this is acting as a decompression, thereby relieving the pressure in the mediastinum. If the air does not disperse itself as described above, the pressure will rise in the mediastinum and tend to compress the large veins. This will interfere with filling of the right side of the heart and obvious distention of the neck veins will occur. Simply marking on the skin each day the progress of the emphysema is a sufficient guide. If the neck veins become distended, and there is no increase in subcutaneous emphysema, then a mediastinotomy is in order. Mediastinotomy carried out just medial to the lower end of the sternomastoid muscle entering into the superior mediastinum will usually suffice.

MASSIVE PULMONARY COLLAPSE AND CONTRECOUP INJURY

Massive pulmonary collapse occurring as a result of trauma to the chest may involve all the lobes of one lung, but more often it involves one, or at most two, lobes. This condition may also result from trauma to the abdomen. In either trauma to the abdomen or chest the actual amount of trauma may be relatively insignificant. Collapse may occur from pressure effects of bomb or shell explosives. In cases of direct trauma or pressure effects of explosions, a contrecoup collapse may occur with only minor involvement of the homolateral side. The mechanism of massive pulmonary collapse is obscure. One theory is that it is a reflex inhibition of movement of the chest wall with interference of the usual aeration.[47]

Clinically, the patient will present far more dyspnea than one would expect from collapse of one lobe. Cyanosis even though more than expected is not as

marked as the dyspnea. The trachea is found deviated to the side of the collapse. Cardiac dislocation toward the affected side will usually be found. Sudden rise in temperature from normal to 103° or 104° F. is also a common finding. Physical findings of a nonaerating lobe or lobes will be found. Following spontaneous aeration or in areation following treatment, râles will appear, and in spite of roentgenologic evidence of good aeration and disappearance of the dyspnea and cyanosis the râles may remain for several days.

Treatment is directed toward reexpansion of the involved lobes. Use of carbon dioxide is recommended to increase the depth of respiration. The patient should be turned with the involved side up and made to cough at five- to ten-minute intervals. If coughing is weak and ineffectual, intubation may be carried out without moving the patient from the bed. A curved semirigid rubber catheter (McGill) may be inserted directly into the trachea, and sudden suction of considerable intensity should be applied to the catheter. During the intubation the patient will often cough strenously enough to aerate the lobes involved. If these measures fail, then bronchoscopy should be carried out to remove mucus from all bronchial orifices. Such endoscopy should be done under local anesthesia and without anesthetizing the vocal cords or trachea. In a surprisingly large number of cases the collapse will disappear following bronchoscopy wherein only small amounts of mucus are aspirated from orifices of bronchi to the involved lobes. It is imperative that treatment be immediate and energetic. Permitting such a collapse to persist will, in the majority of cases, result in pneumonic consolidation. If there is any question of the persistency of the collapse, prompt use of sulfonamide drugs will often prevent an otherwise serious pneumonia. It is felt that the majority of postoperative pneumonias are preceded by some degree of similar collapse.

BOMB BLAST ("BLAST LUNG")

Mass bombing with high explosives has accentuated the importance of blast injury to the lung. Zuckerman and his coworkers,[53] through an extensive series of experiments, have shown that the injury is due to the direct impact of the wave upon the chest wall. Lesions have occurred even when the suction component was excluded.

Experimental animals subjected to the detonation of high explosives show the following sequence of events: There is an initial fall in blood pressure which is ascribed to shock from trauma. Those animals who survive this initial shock, but develop pulmonary lesions, reveal a lowered blood pressure and marked dyspnea. These effects are due to the parenchymal changes, which result from the sudden compression of the chest wall by the wave of high pressure, which simultaneously retards expulsion of the air through the trachea. As a result, the delicate alveolar tissues are ruptured. At the same time the reserve of the pulmonary capillary bed is reduced.[37] Wearn has shown that reduction of this, especially when accompanied by an increase in the blood volume, results in an increase of the intrapulmonary pressure and subsequent fall in the systemic pressure. If the available capillary reserve is reduced to the point where it is insufficient for even the resting venous return, pulmonary edema will result.

Pathologically, the most persistent finding in these animals was multiple areas of hemorrhage in the parenchyma of the lung.[54] These hemorrhagic areas

are most common along the line of ribs, suggesting that these have been driven forcibly against the lungs with resultant contusion. Microscopically, the typical lesions consisted of rupture of the alveolar wall, with hemorrhages originating from the torn alveolar capillaries. The alveoli and smallest bronchioles were filled with blood. Where hemorrhage was severe, frequently there was collapse of the corresponding lobe.

The findings in clinical cases of blast injury are very similar in all respects to those of the experimental animals. Many patients who died had no evidence of external injury and just showed blood-stained froth in the mouth and nose. Post-mortem examination revealed the pulmonary hemorrhages and torn alveolar structures. In patients who have survived the initial blast, there is evidence to indicate that there occurs a diapedesis of red blood cells from intact capillaries which is progressive and lasts for as long as fifty-one hours.[23, 24] Those patients who survived presented the following clinical features: Shock was evident in all and profound in many. Dyspnea was universal, but in many cases did not become very evident until forty-eight to seventy-two hours following the injury.[13] Cyanosis was present in all cases and was proportionate to the degree of injury. Chest pain was of two types, a constant, deep central type and lateral pain which was associated with the respiratory movements.[42] Abdominal pain and rigidity was the predominant finding in some cases, but each case subjected to laparotomy failed to disclose serious intra-abdominal injury. Hemoptysis was not frequent, but blood-stained sputum was common. Cough and expectoration were absent in the first twenty-four hours but invariably appeared thereafter. Rupture of the eardrums was practically pathognomonic.

Physical signs were very meager. A universal finding was the "ballooning of the lower part of the chest."[42] The thorax was apparently one-half to three-fourths distended and fixed in the position of inspiration. Percussion resonance was usually normal. Breath sounds tended to be distant and râles were variable. X-ray examination was of great help in establishing the diagnosis. The typical picture consists of bilateral mottling of the lung fields, resembling a patchy bronchopneumonia, which usually clears in seven to ten days.[42]

It should be noted that in bombing casualties, not all persons in whom there is no external evidence of injury die from the direct effects of blast. Indeed, the English observers emphasized that more people die from such indirect effects as asphyxia, carbon monoxide poisoning, injuries sustained from being trapped in collapsing buildings, and injuries from missiles. The diagnosis of true blast injury of the lung rests upon a reliable history, the presence of shock in the absence of any external injuries, and the development of serious respiratory difficulty.

Treatment of this condition is based upon the foregoing observations. Absolute rest is imperative and should be continued until x-ray examination reveals complete clearing of the lung fields. Oxygen is essential in most cases. In the presence of venous congestion and pulmonary edema, venesection may be a life-saving procedure. Abdominal laparotomy should not be undertaken unless positive proof of intra-abdominal injury is obtained. Any operative procedure should be delayed as long as possible. Transfusions and intravenous fluids are definitely contraindicated. Zuckerman has shown that chests which are well

padded are protected from blast injuries.[54] This obviously proves the necessity for air raid shelters. In the absence of such protection one should assume the prone position, especially behind some substantial structure.

ECCHYMOTIC MASK

A unique condition is "ecchymotic mask," a bluish-violet discoloration of the upper portion of the face and neck extending downward to include the upper portion of the chest which results from sudden direct compression of the chest or abdomen. An excellent consideration of this subject has been written by Heuer.[27] In civilian life it has most often been seen following compression of the chest between the rear end of a truck and a loading platform. Sudden direct compression of the extremities while the patient falls, or is thrown with thighs flexed on the abdomen directly, transmitting the compression to the abdomen, may also give rise to this condition. In wartime such compression may occur from falling debris in bombing with the patient actually crushed. The discoloration is usually more intense at the areas of pressure, such as collar bands, hatbands, and suspenders.

The mechanism is that of marked venous stasis in dilated but intact peripheral venous capillaries. There is no evidence of diapedesis. The intact vessels and absence of extravasation found microscopically, and the absence of usual shades of color occurring as an ordinary ecchymotic spot fades is proof of the stasis in intact vessels. The discoloration may also be found in the pharyngeal and buccal mucosa. In these regions, as well as in the conjunctiva and retrobulbar regions, actual hemorrhage may occur.

Clinically, the discoloration starts to fade at about the second day and usually disappears in two weeks. As a rule, patients are not uncomfortable and the majority clear up without sequelae. There may, however, be ocular symptoms, ranging from mild blurred vision to complete blindness. The retrobulbar hemorrhage, or fundal hemorrhage, may result in permanent optic nerve atrophy. Fortunately, ocular involvement occurs only infrequently.

CARDIAC CONCUSSION

Bright and Beck[10] and Warburg[52] have presented excellent reviews of this subject and have come to the following conclusions: Trauma to the intact chest may be indirectly transmitted to the heart. This is especially true in the young adult, for the chest is far more resilient and flexible than in an older person, permitting the striking force to be more damaging to the heart. The type of injury may be a direct blow over the precordial region, anteroposterior compression of chest between two solid objects (as described in section on ecchymotic mask) or a blow to the abdomen causing sudden rise in intra-abdominal pressure. As can be seen from the above types of injuries, one must be on the lookout for cardiac involvement in any compression injury.

Sufficient experimental work has been done to explain the pathologic physiology. Trauma may cause an actual immediate rupture, and death ensues promptly. The majority of cardiac injuries reported which resulted in death consisted of immediate rupture of one of the chambers. Actual contusion may occur, with hemorrhage and softening and eventual cardiac rupture. The contusion with resultant petechial hemorrhages may commonly involve any portion

of the conduction system. However, the most frequent injury is that causing a reflex spasm of the coronary vessels with a syndrome not unlike that of the common coronary occlusion seen in civilian life causing infarction. Immediate hemorrhage near a coronary vessel or conduction system may interfere with the lumen of the vessel or the functioning of the conduction system, respectively.

It has also been proved experimentally that in case of vagosympathetic imbalance or in a heart sensitized by adrenalin cardiac irregularities may not only occur but coronary spasm may more readily result. Therefore, in the heat of battle with the vascular system surcharged with considerable adrenalin slight trauma to the chest may cause considerable cardiac disturbances.

Clinically, the patient may be dazed or unconscious. If there is no immediate rupture, the patient may rapidly recover and have no subjective symptoms for a period of from several hours to several days, at which time precordial pain or discomfort may develop as a result of vascular occlusion, or the usual dyspnea and choking sensation of cardiac irregularities may ensue.

Clinically, a wide variety of syndromes may be expected. In cases of delayed cardiac rupture from actual contusion there may be a period of one to two weeks from time of injury to sudden development of a cardiac tamponade. In instances of hemorrhage in, or adjacent to, the conduction system a number of cardiac irregularities may result. Ectopic contractions, auricular fibrillation, and auricular flutter may occur. Various types of heart block have been reported. The latter have been found in absence of any marked demonstrable cardiac damage.

The basis of treatment in this type of injury is the recognition of the latent interval between injury and actual infarction and between injury and cardiac rupture from contusion. In all cases of injury to the chest as described above a regime of rest and sedation should be carried out and continued as in cases of coronary occlusion. Absolute and complete rest must be continued for a minimum of at least three weeks. In those cases of chest injury wherein cardiac irregularities are detected then a suspicion of cardiac injury becomes a certainty, and complete and continued rest is essential. Surgical intervention in case of rupture after the latent period is not as successful as in cases of penetrating injuries to the heart, because in cases of rupture due to vascular occlusion the area involved is much larger and adjacent muscle is usually very friable.

PENETRATING WOUNDS OF THE HEART AND PERICARDIUM

Penetrating wounds of the heart occur relatively rarely in civilian practice. This statement is based on Bigger's[5] report of such cases constituting only 0.1 per cent of the surgical cases at the Medical College of Virginia Hospital and Boland's[8] report of 1.4 per cent of 1.187 cases of penetrating wounds of the chest. These statistics represent only those patients who live to reach a hospital and do not represent the true total number of cases. Many probably die promptly from an extremely rapid tamponade because the wound of the myocardium is so placed as to cause massive bleeding.

In instances of bayonet wounds or wounds caused by small caliber bullets, the hemorrhage into the pericardium may be such that the cardiac tamponade will occur slowly enough to permit surgical closure. Cardiac tamponade presents

a syndrome of a progressive dropping of systolic pressure, rising venous pressure with distended neck vein, quiet distant heart tones, and increase in area of cardiac dullness. Repeated aspirations of the pericardium may be done to tide a patient over until surgical closure is accomplished. Aspiration of the pericardial cavity may be accomplished by inserting a needle in the angle between the xyphoid process and the adjacent left costal arch. The needle is directed cephalad inward and toward the left at approximately an angle of 45 degrees. Surgical repair may be accomplished through a curved incision exposing the third, fourth, and fifth costal cartilages, resection of sufficient portions to expose the pericardium, opening of the pericardium, and suture of the wound of the heart. It is essential to use positive pressure anesthesia. Technique of such closure has been fully described by Elkin[15] and Beck.[1]

CARDIAC RESUSCITATION

The problem of cardiac standstill, formerly assumed to be a problem handled only by the surgeon in a meticulously fitted operating room, is now because of war casualties a problem of all physicians wherever and whenever it occurs. Cardiac cessation results either from failure of the sinus node or as an end result of ventricular fibrillation. By far, the former is the most common cause. The idea of reviving the human heart, when its action is stopped, is feasible, as shown by Koontz,[36] who by perfusion experiments revived 65 of 127 hearts, whose action had stopped for a period of two hours. Forty-eight of these 65 (73.8 per cent) developed a regular heartbeat.

An outline for resuscitation is suggested as follows:

I. Essential factors in treatment of all cases
  - A. Unobstructed airway
  - B. Artificial respiration
    - 1. Manual
    - 2. Mouth-to-mouth insufflation
    - 3. Mechanical respirators

II. With intact abdomen and chest
  - A. Blow to precordium
  - B. Direct stimulation of heart
    - 1. Needle in right auricle
    - 2. Injection of epinephrine
    - 3. Electrical stimulation via the needle
  - C. Massage of heart
    - 1. Through emergency laparotomy, massage conducted through an intact diaphragm
      - (a) Massage through an opening of the diaphragm

III. During thoracotomy
  - A. Massage of the heart
  - B. Use of electrical stimulation or drugs, as indicated by topical application

IV. During laparotomy
  - A. As under II. C

In any treatment of cardiac resuscitation the maintenance of proper oxygenation is imperative. Irreversible cerebral damage can occur within five to eight minutes in the presence of anoxemia. Proper oxygenation can be achieved and maintained only by assuring the immediate institution of an unobstructed airway and artificial respiration.

The problem of an unobstructed airway is essential to proper oxygen exchange. Removal of foreign body obstruction in the upper respiratory tract with the jaw held up and forward will usually suffice. The unconscious patient will not often present a spasm of the glottis, but if this does occur intubation is essential.

Artificial respiration should be started immediately. This may be accomplished by the Schaefer prone method, mechanical respirators, or even mouth-to-mouth insufflation. The important fact is that one should not await the assembling of an armamentarium sufficient to cope with the condition before starting artificial respiration. The mechanical expansion and collapse of the lungs directly helps to stimulate the heart. This has been clearly demonstrated by Thompson, Birnbaum, and Shiner[49, 50] by the phenomena of "asphyxial resuscitation." The mechanical respirators, utilizing a positive and negative pressure, are excellent when available, and if intubation is necessary, complete control of respiration is assured.

In cases of cardiac standstill occurring with an intact abdomen and chest, as soon as an obstructed airway and artificial respiration are obtained, sharp blows to precordium may induce cardiac contraction (Cordelli[12]). This is, of course, unreliable, but may be done quickly while other measures are being started. Immediate stimulation of the right auricle by a needle is next in order. Mere mechanical stimulation of the right auricle may induce contraction. It is important that the irritation occurs in the auricle. Hyman[29, 30] has shown that the "action current of injury" may produce flutter followed by fibrillation. As fibrillation of the ventricles is conceded incompatible with life, it is, therefore, necessary that the needle be inserted in the region of the right auricle. A needle about 4 or 5 inches long inserted in the third or fourth intercostal space at the edge of the sternum, directed inward and slightly caudad, will approach the approximately correct position. If mechanical irrigation does not succeed, then injection of 1 c.c. of 1:1,000 epinephrine into the right auricle is next in order. The institution of all the above can be accomplished in from eighty to one hundred and twenty seconds. Next, electrical stimulation utilizing principles as elucidated by Hyman[31] and Beck[2] may be conducted per needle. Application of electrodes directly to the heart are, of course, far more efficient. Use of ordinary electric light current, with a resistance coil to decrease the amperage to one or one and one-half amperes, may be used.

If all these measures fail, at the end of eight minutes, then massage of the heart should be instituted. Opening of the abdomen through an incision in the epigastrium large enough to admit a hand should be accomplished promptly. The patient is to all intents and purposes dead and will most assuredly remain so unless cardiac action is started. Massage is then done by pushing the heart against the anterior chest wall through the intact diaphragm. If no contractions have occurred after sixty seconds, the diaphragm and adjacent pericardium

should be opened, and with the thumb directly on the heart, and the fingers outside the diaphragm, good massage can be accomplished. This method, with the technique of opening the diaphragm just behind the xyphoid process, between the attachments of the two sides of the diaphragm through the avascular area is described by Nicholson.[41]

In instances of cardiac standstill during thoracotomy, direct massage of the heart is by far the best method.[2, 30] Application of Hyman's pacemaker,[31] or Beck's electrodes[2] can be used under direct vision.

The problem of ventricular fibrillation is essentially one of stopping cardiac motion by shocking it into standstill, and then reshocking it into a more normal action.[2, 30] Calcium gluconate intravenously may be used to decrease the sensitivity of the heart, and sodium iodide may be used intravenously to increase the sensitivity, with resultant cardiac motion.

The foregoing suggested outline of treatment may be utilized at advanced medical installations. In all cases of casualties, without obvious cause of death, and with no discernible heart action, immediate energetic action may truly be lifesaving. The mere establishment of an unobstructed airway, institution of artificial respiration, and mechanical stimulation of the right auricle with a needle, may, if done promptly, revive seemingly hopeless patients.

ACUTE EMPYEMA

Acute empyema during the early part of the influenza epidemic of 1917-1918 treated by early open drainage carried an average mortality of 30.2 per cent.[18] In some camps it reached 90 per cent.[44] As a result of the investigation of Graham and Bell,[18] certain principles were elucidated, and treatment of empyema based on those principles reduced the mortality to 4.3 per cent. In view of present war conditions and the possibility of the outbreak of epidemics of influenza with streptococcal pneumonias and resultant empyema, the principles outlined by Graham and Bell should be reviewed, in order that we may not repeat the errors made during World War I.

The principles are: (1) Drainage, but with the careful avoidance of an open pneumothorax during the period of active pneumonia; (2) early sterilization and obliteration of the cavity; and (3) maintenance of nutrition of the patient.[19] The fact that the majority of the patients in army camps died as a result of an open pneumothorax (operative) during the period of an acute pneumonia was the basis for the investigation which resulted in the change in the method of treatment. During the period of acute streptococcal pneumonia, which was so prevalent in the army camps, the vital capacity was markedly lowered, and the patient was then subjected to an additional lowering of vital capacity by the establishment of an open pneumothorax. When this plan of treatment was followed, the mortality was exceedingly high. As a result simple aspiration during the pneumonic stage was substituted.[18] This was done as often as every three to four days. However, dyspnea from accumulation of large amounts of pleural fluid must not be permitted to occur. When the patient has recovered from the stage of acute pneumonia and the empyema is localized in one portion of the pleural cavity and the pleural fluid removed is frank pus, open drainage can be safely performed. By this time, the vital

capacity has greatly increased. The changing character of the fluid is a good index of what is occurring, and when it has definitely changed from a thin, slightly cloudy fluid to frank, thick pus, one may rightly feel that there is a considerable stiffening of the mediastinum and adequate drainage of the empyema should be accomplished.

Both the intercostal catheter drainage and drainage by rib resection have their advocates, and satisfactory results are obtained by the proponents of each method. The important consideration is the appreciation and application of the principles involved in the proper treatment of acute empyema rather than dogmatic statements about a particular method. The avoidance of an open pneumothorax during the acute stage of the pneumonia is of paramount importance. Adequate drainage of the localized empyema must then be instituted.

Obliteration of the cavity is a fundamental principle of treatment. Chemical sterizilation by irrigation every two to four hours with Dakin's solution liquefies the necrotic material and permits it to drain more readily. No chemical can sterilize a cavity without completely free drainage. Recently, it has been shown that the addition of solutions which lower surface tension to Dakin's solution, aids the latter in the liquefying process and tends to enhance its sterilizing qualities. Such detergent substances have been described by Petroff and Schain.[43] The cavity should be measured daily, if a bronchopleural fistula is not present, and the tube must not be removed until the cavity measures less than 10 c.c. The removal of the tube before the cavity is obliterated is an important cause of the development of chronic empyema. The maintenance of the nutrition of the patient by the intake of a high calorie and high vitamin diet supplemented by blood or plasma transfusions is essential.

CHRONIC EMPYEMA

Chronic empyema must of necessity be mentioned in any discussion of war injuries, for the majority result from ill-timed and poorly planned early treatment. Keller,[33] in reporting on a large series of chronic empyema following World War I, found that 75 per cent had accessory pockets, 15 per cent had foreign bodies (drainage tubes and bismuth paste), and 90 per cent had osteomyelitis of the ribs. These were all residual cases in which hemolytic streptococci were found. Too early open drainage was done in a vast majority of instances instead of awaiting localization of the empyema. Many cases, therefore, suffered a total empyema to start with, and of these the majority had inadequate drainage so that pockets developed and remained. If the principles of treatment as outlined in the section on acute empyema are followed, there will not be a large group of chronic empyemas following World War II. For detailed consideration of chronic empyema, the reader is referred to the work of Keller[33] and Graham.[19]

FOREIGN BODIES

In any operative procedure wherein the pleura is explored, all foreign bodies should be removed. In the emergency treatment of sucking wounds of the chest, the removal of foreign bodies should be secondary to immediate strapping of the wound. In such instances the foreign body can be removed during the surgical closure.

In general, foreign bodies in the parenchyma should be removed. However, the mere presence of a foreign body in the parenchyma is not alone an indication for its removal. Foreign bodies lodged in the pulmonary parenchyma in patients who have recovered and are apparently well should be studied carefully in base hospitals, and the treatment must be individualized. As a rule, if the foreign body is a single bullet, there is less chance of it eventually causing complications, than if the foreign bodies are shrapnel, shell fragments, or multiple splinters. Retained foreign bodies often result in late pulmonary suppuration.

In any condition causing prolonged pulmonary suppuration, surgeons should become foreign-body conscious. Drainage tubes are the most frequent offenders. Tubes should be pierced with a safety pin, so that if red rubber radio-opaque tubes are not available, the tube can be located by x-ray. With this in mind, it is well not only to fix drainage tubes securely, but also to state clearly in his medical record the fact that such tubes are used on a person.

COMBINED ABDOMINOTHORACIC WOUNDS

Combined wounds of both thorax and abdomen are not infrequent and they carry a high mortality. In World War I this type of wound was found in 4.6 per cent of all chest injuries.[28] In the Spanish Civil War these wounds constituted 11 per cent of all abdominal wounds. Heyd[29] reported a mortality of 60 to 75 per cent during World War I. From reports of World War I by Gordon-Taylor,[17] prognosis is usually worse if the point of entrance is in the abdomen. Penetration of a hollow viscus, hemorrhage from the spleen or liver, or evisceration are the most common grave possibilities.

The treatment of simultaneous wounds of the chest and abdomen often present a difficult problem. It is desirable to close the thoracic opening before the abdomen is explored through the abdominal wound. This permits the correction of disturbed cardiorespiratory physiology, and the patient's vital capacity will be increased. It is to be remembered that in wounds of the chest the upper abdomen may be spastic and pain may be referred to the abdomen without any involvement of the abdominal contents.[3] In the closure of such wounds it is important to trace the course of the bullet and to inspect the diaphragm. It may be that there is an open wound of the chest with a rigid but intact abdomen in which the contained viscera is involved and the bullet remains in the abdomen. In such cases exploration of a large portion of the abdomen can be conducted through the thoracic wound by merely enlarging the opening in the diaphragm. Certain wounds of the stomach, liver, and intestines can be managed through such an approach. The repair of diaphragmatic tears is essential. The replacement of abdominal organs herniated up into the chest through the diaphragmatic opening helps restore the proper cardiorespiratory physiology. Various techniques for closure of diaphragmatic hernias, as described by Schiffbauer,[45] Harrington,[25] and Truesdale[51] may be used.

CHEMOTHERAPY

Wounds of the chest, like wounds in any region of the body, will respond, to a large degree, according to whether or not infection supervenes. One of the epochal events in medical progress since World War I was the introduction of

the sufonamide drugs. Recent studies have proved this remarkable series of dye derivatives to be valuable in the prevention of infection as well as in the control of sepsis, and to have a local bacteriostatic effect that makes them of paramount importance in the prophylaxis of wound infections. In the present war the role of the sulfonamide drugs in this respect may transcend anything we are prepared to believe with our present limited perspective based only on the more or less ideal surgical circumstances of peacetime experience. A consideration of the sulfonamide therapy of war wounds of the chest, therefore, necessitates a discussion of both the systemic and the local application of the drugs. For the present, the accepted representatives of the sulfonamide group for surgical purposes are sulfanilamide, sulfathiazole and sulfadiazine.

The remarkable reduction in infection rate in a series of compound fractures, published by Jensen and others in 1939,[32] treated by the local implantation of sulfanilamide, marked a new era in the treatment of wounds, and opened an entirely new vista for this amazing drug group. Their results were quickly corroborated by others. Key and his group[34, 35] carried out experiments, proving that the drug has no deleterious effects on the tissues, and does not delay wound healing. Experience was consistent in acknowledging the marked decrease in wound infections when sulfanilamide was introduced into the wounds. Intrathoracic wounds, however, produced a unique set of circumstances. Here, as in no other region, large, unobliterable cavities are likely to be encountered, and more important, one frequently has to deal with the "open bronchus," which serves as a perpetuating source of virulent, drug-refractory anerobic pathogens. Cognizant of these difficulties, Burford and Graham[11] implanted the drug in a series of total pneumonectomy and pulmonary lobectomy cases. It was found, as expected, that the drug did not prevent the development of an empyema, if the bronchus opened, but that the streptococcal component of the bacterial flora was inhibited and frequently eradicated, and for that reason perhaps the severity of the process seemed frequently less severe. There was some indication that contamination of the pleural space at the time of operation was less likely to result in empyema, if the drug was used, provided, of course, the bronchus did not open. No damage to the pleura or pericardium occurred, and very high hemic concentrations of the drug were promptly obtained.

The exigencies of war surgery will of necessity frequently leave much to be desired from the standpoint of impeccable technique, and trustworthy asepsis. It is here that the sulfonamides will doubtless prove invaluable. It is our belief that any chest wound whether intrapleural or of the chest wall alone should have the benefit of this added precaution, sulfonamide locally, even though contamination is known to be minimal. Never, however, should the anticipated use of the drug entice one into a false sense of security, and a consequent lowering of the standards of proper wound handling.

Whether sulfanilamide or sulfathiazole should be used is a matter that would be easier to decide if one could be sure of the invading microorganism. Sulfanilamide is probably more efficacious against the streptococci, and sulfathiazole is certainly more lethal to the staphylococci. Some prefer to use a mixture of equal parts of sulfanilamide and sulfathiazole.

The amount of drug used will vary with the type and extent of the wound. For example, one gram would be sufficient for a laceration of one or 2 inches of the chest wall and not involving the pleural cavity, while as much as 12 or 15 Gm. could be placed with impunity in the space left by a total pneumonectomy or pulmonary lobectomy. In using larger amounts, it is to be remembered that the drug is absorbed very rapidly, particularly from the pleural space, and high blood levels are reached very quickly. If a large dose is implanted, the development of a methemoglobinemia should be anticipated and properly dealt with.

All those experienced in the local use of the sulfonamides are agreed that it in some unexplained fashion tends to increase the likelihood of bleeding and hematoma formation. Particular care must, therefore, be taken to achieve completely adequate hemostasis. In this regard, it is mandatory to remember that it is usually unwise to introduce the drug into the pericardial cavity, unless contamination has been excessive. Not only does the drug frequently occasion disturbing alterations in cardiac rhythm when dusted on the epicardium, but its use is almost certain to occasion a collection of fluid which may cause a serious degree of cardiac tamponade. If the drug is placed in the pericardial cavity, drainage is obligatory. In all cases wherein large amounts of dye are placed in the pleural cavity, the resultant pleural pressure changes must be carefully observed and promptly adjusted. These dyes act as hypertonic solutions, causing the development of pleural effusions. When the pleural cavity is not drained postoperatively, the occurrence of effusion with accompanying mediastinal shift may cause dyspnea proportional to the amount of the effusion. Aspiration of a sufficient portion to permit the patient to breathe comfortably is indicated. It is unnecessary to empty the chest completely for return to normal dynamics can be effected with removal of only 200 to 300 c.c., when there may be a total of as much as 2,000 c.c. By this method, the majority of the fluid remains with its high concentration, and the effectiveness of the drug is utilized without deleterious pressure effects.

The sterilization of the drug previous to implantation into wounds has been the subject of considerable discussion, and while sterilization is highly desirable, it is not absolutely necessary, and we have no hesitancy in using the unsterilized powder if the facilities for autoclaving are not available. Most of the commercial preparations are now supplied in sterile ampoules.

Once infection has supervened, then, of course, the systemic role of the drugs must be relied upon. In infected wounds of the chest, sulfonamide is promptly indicated in adequate doses just as in infection elswhere. In this regard, we wish to emphasize that the continuance of sulfonamide therapy with the neglect of proper surgical drainage, once pus has formed, can only lead to disaster.

We are certain that the prophylactic administration orally of sulfonamide, preferably sulfathiazole, will do much to minimize or to prevent the development of pneumonitis in cases of pulmonary atelectasis, which are not promptly reaerated. These cases are not infrequently associated with severe chest trauma. It is our practice to give sulfathiazole promptly in all such cases when there is the least doubt that complete pulmonary re-expansion has occurred.

ANESTHESIA

Anesthesia employed in thoracic surgery in wartime may be considered in relation to its use in intrapleural and extrapleural surgery, as well as to its availability in various army installations.

The following can be used in extrapleural surgery: local (novocain), sodium pentothal, ether, nitrous oxide, and oxygen with or without ether and cyclopropane. All the anesthetic agents, except cyclopropane, will be available at evacuation hospitals. Cyclopropane will probably be reserved for the numbered and named general hospitals.

The anesthetics, which can be employed in intrapleural surgery are: cyclopropane, nitrous oxide and oxygen, ether, sodium pentothal, and spinal nupercaine (1:1,500). Nitrous oxide, oxygen, and ether, and sodium pentothal will be used as near the front as necessity requires. Nupercaine (1:1,500) or Jones solution has received more enthusiastic acclaim by the British.

While a trained anesthetist is a boon to the abdominal and orthopedic surgeon, he is an absolute necessity to the thoracic surgeon. A general knowledge of the anesthetic agents and methods of administration usually suffices for abdominal and orthopedic surgery. At least, it may be stated that with a minimum of knowledge untrained personnel may administer anesthetics for abdominal and orthopedic surgery in the press of war. However, anesthetists administering anesthesias in intrapleural surgery must have not only thorough knowledge of the physiology of respirations but also sufficient experience to cope with the many disturbances of the cardiorespiratory mechanism which may occur.

In considering premedication, morphine and scopolamine are the drugs of choice. Many patients will have received morphine sulfate during the first aid treatment and this must be kept in mind in any further premedication. We prefer scopolamine to atropine sulfate because the former has a sedative action that the latter lacks. Soldiers who have had a large meal before trauma or shock occurs, will, because gastric motility is decreased, retain the food in an undigested state. Lavage may be necessary before the anesthetic is started.

Nerve block with novocain can be used in extrapleural procedures. The pain of fractured ribs can be relieved by intercostal nerve block.[46] Large extrapleural wounds are readily managed by local injections. Simple rib resections are easily accomplished under novocain anesthesia. Gas casualties with chest wounds can be readily managed by local anesthesia.

Intravenous sodium pentothal is now a well-established method and forms a valuable adjunct to the anesthetist's armamentarium. Gas anesthesias are widely used. Ether is the most universal. Nitrous oxide, oxygen, and ether makes possible adequate oxygenation by the reduction of the concentration of the nitrous oxide used. It is otherwise impossible to control robust men without some form of anoxia when nitrous oxide and oxygen alone are used. Cyclopropane is particularly adapted to chest surgery because it is given with a high concentration of oxygen. Cyclopropane has nearly replaced ethylene, which is lighter than air and does not give the depth of anesthesia that is possible with cyclopropane.[22]

Proper endotracheal anesthesia has made possible the modern advances in intrapleural surgery. With a carefully developed technique the anesthetist can completely control the patient's entire respiratory cycle and can maintain the proper intrapulmonic pressure at all times.[21] A further very important consideration is that in cardiac standstill the primary factor in cardiac resuscitation is satisfied. Adequate oxygenation through an open airway is thus assured. The special advantages of such a closed system are that it regulates intrapulmonary pressure, controls respirations, and conserves body heat.

Once the chest has been opened, pressures of from +6 to +8 cm. of water are necessary.[40] This maintains sufficient inflation to produce a vital capacity which is above a dangerous level. The amount of pressure should not be a matter of approximation but should be carefully checked by a manometer. The water manometer of Flagg or the graduated weights of Connell are excellent. If these are not available, a simple manometer may be constructed by inserting a measured length of tube into a column of water (Barach). This prevents the raising of the intrapulmonary pressures beyond dangerous limits, even though the bag may be suddenly and forcibly compressed by the anesthetist.

One of the most recent developments has been the use of controlled respirations.[21] It is a great boon to the operator if the patient is under the control of the anesthetist. The rate and depth of respirations can be regulated and stopped and started at the will of the anesthetist. During repair of the diaphragm, manipulation of the mediastinal structures, and suture of the parenchyma of the lung, the movement of the lung may be stopped for a period sufficient to do careful minute work. The technique of controlled respirations, as introduced by Guedel,[21] is reported by Neff and associates for use in thoracic surgery.[40]

In front line surgery it is conceivable that intratracheal anesthesia will not be available. In such instances a tightly fitting face mask will be of value. Any mask with an inflatable cushion will be efficacious (Connell's or McKesson's).

Cyclopropane and oxygen is the anesthesia of choice in intrapleural surgery. The principal advantages of cyclopropane are the high oxygen content, the ease of induction, and the lack of irritation to the lungs and other organs of the body. In our experience there has been little effect on the blood pressure. A rise of over 20 mm. of mercury is the exception. The one disadvantage of cyclopropane is its explosive quality. This is mentioned because it has been overemphasized. Greene states that "Under the same clinical circumstances ether and oxygen, with or without nitrous oxide has the same great tendency toward propagating a wave of flame or pressure through the respiratory tract as has ethylene-oxygen and cyclopropane-oxygen."[20] This is confirmed by the report of the committee appointed by the American Society of Anesthetists to investigate the hazard of fires and explosions due to anesthetic agents. They emphasized that nitrous oxide oxygen and ether is just as explosive as cyclopropane. The principal advantages of ether and air are that it has a relatively small tendency to propagate a wave of flame or pressure down the respiratory tract, even though explosions of ether and air can cause death. By the closed method of administration small amounts of ether are necessary for anesthesia so it is less irritating. This is particularly true when ether is used to *supplement* nitrous oxide and oxygen. Addition of a small quantity of ether enables the anesthetist to increase the oxygen percentage in the mixture. Ether alone or in

gaseous mixtures gives the untrained personnel a large margin of safety so that it is relatively safe. However, ether is still unpleasant to the patient and the induction period is longer than with gases.

Sodium pentothal intravenously with 100 per cent oxygen by the intratracheal McGill tube is a more recent idea in thoracic surgery. Sodium pentothal has been used extensively, and even the most ardent advocates of nitrous oxide are now using sodium pentothal. After the endotracheal tube is inserted, a cocaine spray through the tube is necessary to abolish the bronchial reflexes initiated by the operator's manipulations. It is always advisable to administer oxygen to the patient under sodium pentothal, since there is a gradual depletion of oxygen in the circulating blood.

Regardless of the type of anesthesia, oxygen by the nasal catheter introduced into the oropharynx should be continued postoperatively for a few days. While intravenous fluids are given from the beginning of the operation, plasma and whole blood will be indicated postoperatively; if plasma or whole blood has been given during the operation, they may have to be repeated. Of course, the patient should be kept comfortable with morphine. This should be ordered as the indication arises. At no time should the respirations or cough reflex be allowed to become too depressed by medication. Carbon dioxide by inhalation is given in the treatment of atelectasis.

SUMMARY

The proper management of thoracic war injuries is a timely subject. The mortality of such injuries has always been high and many persons surviving such injuries have suffered prolonged chronic illness due to extrapulmonary or intrapulmonary complications. In order to decrease these hazards it is necessary that the medical officer called upon to treat such injuries understands the basic principles of the abnormal thoracic physiology resulting from chest wounds. The methods of treatment outlined in this paper are based upon these principles.

The first essential in the management of thoracic war injuries is the immediate care of such wounds. This requires the closure of sucking wounds, control of hemorrhage, and relief of a tension or pressure pneumothorax. This immediate treatment must be followed by early definitive treatment supervised by personnel qualified in thoracic surgery working in properly equipped medical installations where casualties with chest wounds are concentrated within a few hours after the injuries are incurred. All subsequent treatment must likewise be supervised by competent thoracic surgeons.

The treatment of shock in chest injuries requires the same close attention as in other injuries producing shock. It is imperative, however, that immediate attention must be given to correcting the abnormal thoracic physiology. The sources of hemorrhage and the methods of controlling such hemorrhage has been detailed. A wound producing an open pneumothorax or sucking wound of the thorax must be immediately closed. Concurrent and later therapy must conform to the principles outlined in correcting the abnormal physiology. A tension pneumothorax must be relieved. A simple method for accomplishing the decompression is by introducing a large bore needle into the corresponding pleural

cavity, preferably in the second intercostal space anteriorly 2 inches lateral to the sternal border. Care must be observed to prevent obstruction of the lumen of the needle. The recognition of massive pulmonary collapse and treatment directed toward re-expansion of the involved pulmonary tissue will greatly lessen the complications which frequently follow this condition. The phenomenon of bomb blast or "blast lung" has been discussed with a view of enabling its recognition and of preventing unnecessary surgical intervention. The management of cardiac concussion, cardiac wounds, and a rather detailed presentation of cardiac resuscitation have been described. The importance of an understanding of the fundamental principles underlying the proper treatment of acute empyema has been stressed. Foreign bodies in the pleural space should always be removed as early as possible. Many foreign bodies in the pulmonary parenchyma will require subsequent operations for their removal, and each case should be carefully investigated by employing all available aids in the localization of the foreign body before surgical exploration. The sulfonamide drugs should be employed in all thoracic injuries and operative procedures. The anesthetic agents employed in thoracic surgery and the utilization of these agents in war wounds of the chest have been discussed. The recognition of properly employed endotracheal anesthesia will, it is believed, be highly successful in the management of many chest wounds. Likewise the greater use of cyclopropane is considered advisable. Finally, modern thoracic surgery was born during World War I, and has grown to maturity during the past two decades. It is reasonable to predict that its proper employment will contribute to greater developments of this new field of surgery, that many lives will be saved, and that chronicity will be minimized.

REFERENCES

1. Beck, C. S.: Wounds of the Heart; Technique of Suture, Arch. Surg. 13: 205, 1926.
2. Beck, C. S.: Resuscitation of Cardiac Standstill and Ventricular Fibrillation Occurring During Operation, Am. J. Surg. 54: 273, 1941.
3. Bennett, R. H., and Burbank, B.: Acute Abdomen During Pneumothorax Therapy, Am. Rev. Tuberc. 41: 50, 1940.
4. Berry, F. B.: The Treatment of Injuries to the Chest, Am. J. Surg. 54: 280, 1941.
5. Bigger, I. A.: Heart Wounds; Report of Seventeen Patients Operated Upon in Medical College of Virginia Hospitals and Discussion of Treatment and Prognosis, J. Thoracic Surg. 8: 239, 1939.
6. Blades, B.: Emergency Treatment of Traumatic Chest Injuries, Surgical Clin. North Amer. 20: 1473, 1940.
7. Blades, B., and Kent, E. M.: Individual Ligation Technique for Lower Lobe Lobectomy, J. Thoracic Surg. 10: 84, 1940.
8. Boland, F. K.: Traumatic Surgery of the Lungs and Pleura; Analysis of 1,009 Cases of Penetrating Wounds, Ann. Surg. 104: 572, 1936.
9. Bourne, W., Leigh, M. D., Ingles, A. N., and Howell, G. R.: Spinal Anesthesia for Thoracic Surgery, Anesthesiology 3: 272, 1942.
10. Bright, E. F., and Beck, C. S.: Non-penetrating Wounds of the Heart; Clinical and Experimental Study, Am. Heart J. 10: 293, 1935.
11. Burford, T. H., and Graham, E. A.: Local Use of Sulfanilamide in the Pleural Cavity, J. Thoracic Surg. 11: 203, 1941.
12. Cordelli: As quoted by Thompson and co-workers.[50]
13. Dean, D. M., Thomas, A. R., and Allison, R. S.: Effects of High Explosive Blast on Lungs, Lancet 2: 224, 1940.
14. DeBakey, M.: The Management of Chest Wounds, Surg., Gynec., & Obst. 74: 203, 1942.
15. Elkin, D. C.: Emergency Surgery of the Heart, Am. J. Surg. 46: 551, 1939.
16. Foster, J. M., Jr.: Treatment of Lung Penetrations, Tr. West. S. A. 48: 476, 1939.
17. Gordon-Taylor, G.: Chest Surgery in War: Abdomino-Thoracic Injuries, Brit. M. J. 1: 862, 1941.

18. Graham, Evarts, A.: The Medical Department of the United States Army in World War I, Vol. XI, Surg. Part 2. Office of the Surgeon General, U. S. Army.
19. Graham, Evarts A., Singer, J. J., and Ballon, H. C.: Surgical Diseases of the Chest, Philadelphia, 1935, Lea & Febiger.
20. Green, B. A.: Hazards of Fire and Explosions of Anesthetics in Presence of Cautery, Surg., Gynec. & Obst. 74: 259, 1942.
21. Guedel, R.: Cyclopropane Anesthesia, Anesthesiology 1: 13, 1940.
22. Guedel, R.: Inhalation Anesthesia, New York, 1937, The Macmillan Co.
23. Hadfield, G., and Christie, R. V.: Case of Pulmonary Concussion ("Blast") Due to High Explosive, Brit. M. J. 1: 77, 1941.
24. Hadfield, G., and Christie, R. V.: Blast From High Explosive: Preliminary Report on Ten Fatal Cases, With Notes on Identification and Estimation of the Carboxyhaemoglobin in Formal Fixed Material, Lancet 2: 478, 1940.
25. Harrington, S. W.: Diaphragmatic Hernia: Symptoms and Surgical Treatment in 60 Cases, J. A. M. A. 101: 987, 1933.
26. Harrington, S. W.: Diagnosis and Treatment of Various Types of Diaphragmatic Hernia, Am. J. Surg. 50: 381, 1940.
27. Heuer, G. J.: Traumatic Asphyxia, With Especial Reference to Its Ocular and Visual Disturbance, Surg. Gynec. & Obst. 36: 86, 1923.
28. Heyd, G.: Thoraco-Abdominal Injuries: Some Technical Procedures Developed by War, Ann. Surg. 72: 370, 1920.
29. Hyman, A. S.: Intra-auricular Puncture, J. A. M. A. 97: 408, 1931.
30. Hyman, A. S.: Resuscitation of Stopped Heart by Intracardial Therapy: Experimental Use of Artificial Pacemaker, Arch. Int. Med. 50: 283, 1932.
31. Hyman, A. S.: Resuscitation of Stopped Heart by Intracardial Therapy: Further Use of Pacemaker, U. S. Nav. M. Bull. 33: 205, 1935.
32. Jensen, N. K., Johnsrud, L. W., and Nelson, M. C.: The Local Implantation of Sulfanilamide in Compound Fractures: A Preliminary Report, Surgery 6: 1, 1935.
33. Keller, W. L.: The Treatment of Chronic Empyema Where Recognized Surgical Procedures Have Failed to Produce Obliteration, Ann. Surg. 76: 549, 700, 1922.
34. Key, J. A., and Burford, T. H.: The Local Implantation of Sulfanilamide in Compound Fractures. Its Effects on Healing, South. M. J. 33: 449, 1940.
35. Key, J. A., Frankel, C. J., and Burford, T. H.: The Local Use of Sulfanilamide in Various Tissues, J. Bone & Joint Surg. 22: 952, 1940.
36. Koontz, W. B.: Revival of the Human Heart, Ann. Int. Med. 10: 330, 1936.
37. Krohn, P. L., Whitteridge, D., and Zuckerman, S.: Physiologic Effects of Blast, Lancet 1: 249, 1942.
38. Lee, B. J.: Wounds of the Abdomen, The Medical Department of the United States Army in the World War, Vol. 2, Chap. 15, p. 466, Washington, D. C., Government Printing Office, 1927.
39. Morelli, E.: The Treatment of Wounds of Lung and Pleura, Translated by Davis, L., and Irving, F. C., Boston, 1920, W. M. Leonard.
40. Neff, W., Wilson, P., and Gunn, G.: Anesthesia for Pneumonectomy in Man, Anesthesiology 3: 314, 1942.
41. Nicholson, J. C.: Cardiac Massage, Brit. M. J. 2: 385, 1942.
42. O'Reilly, J. N., and Gloyne, S. R.: Blast Injury of Lungs, Lancet 2: 423, 1941.
43. Petroff, S. A., and Schain, P.: Enhancement of Bactericidal Properties of Well Known Antiseptics by Addition of Detergents, Quart. Bull., Sea View Hosp. 5: 372, 1940.
44. Preliminary Report by Empyema Commission, Camp Lee, Petersburg, Va. J. A. M. A. 71: 366, 433, 1918.
45. Roberts, J. E. H., and Tubbs, O. S.: Recent Experience With War Wounds of the Chest, Am. J. Surg. 54: 289, 1941.
46. Rovenstine, E. A., and Wertheim, H. M.: Therapeutic Nerve Block, J. A. M. A. 117: 1599, 1941.
47. Sante, L. R.: Massive Collapse of the Lung, J. A. M. A. 88: 1539, 1937.
48. Schiffbauer, Hans E.: Traumatic Diaphragmatic Hernia: Transpleural Repair, California & West. Med. 50: 180, 1939.
49. Thompson, S. A., and Birnbaum, G. L.: Phenomena of Asphyxial Resuscitation, Proc. Soc. Exper. Biol. & Med. 4: 203, 1941.
50. Thompson, S. A., Birnbaum, G. L., and Shiner, I. S.: Cardiac Resuscitation, J. A. M. A. 119: 1479, 1942.
51. Truesdale, P. E.: Diaphragmatic Hernia; Its Varieties and Surgical Treatment of the Hiatus Type, Am. J. Surg. 32: 204, 1936.
52. Warburg, E.: Subacute and Chronic Pericardial and Myocardial Lesions Due to Non-Penetrating Traumatic Injuries, London, Levin & Munksgaard, 1938, and Brit. Heart J. 2: 271, 1940.
53. Zuckerman, S.: Experimental Study of Blast Injuries to Lungs, Lancet 2: 219, 1940.
54. Zuckerman, S., and others: Discussion of Blast Injuries, Proc. Roy. Soc. Med. 34: 171, 1941.

WOUNDS OF WARFARE*

Frederick A. Coller, M.D., and Jack Matthews Farris, M.D.
Ann Arbor, Mich.

WOUNDS produced by the stone club of prehistoric man or by the tusk of the saber-toothed tiger or mammoth were probably the first pathologic conditions observed by man in which the etiology was known. It is almost unbelievable that we should know so little about them today. A thoughtful reading of the historical aspects of the treatment of wounds is obligatory to a proper understanding of the philosophy of the subject. Without this one cannot well evaluate the modern suggestions for the management of wounds. With every war there is a revival of interest in the methods of caring for wounds, since there is produced a laboratory for observation and experiment. Up to the time of Lister many worth-while suggestions based upon empirical observations were made and discarded. Infection in wounds was not a matter of paramount importance to the early writers on Surgery. Hippocrates, Celsus, Franco, and Paré did not emphasize its frequent or fatal occurrence. It is probable that in their day infection was neither inevitable nor serious.

Serious infection in wounds is first commonly mentioned in the surgical writings of the eighteenth century. It is significant that at this time hospitals had been established in which the wounded were congregated for treatment *en masse.* Accounts of hospital gangrene, erysipelas, and putrid infections then made their appearance in the surgical literature. While many surgeons made important observations on treatment, it remained for the founding of bacteriology by Pasteur and its application to surgery by Lister to bring some rationale to the treatment and prevention of infection in wounds. The students of Lister established the principle of antisepsis that acted potently upon the bacteria found in the air, on the patient, and in the exudates. Carbolic acid and its successors were the forces that were to abolish bacteria from wounds of accident or intention. Through the antiseptic to the aseptic era enormous progress was made and modern surgery originated. Pus remained an emblem of defeat, however, and it was washed, irrigated, drained, and neutralized by a great number of chemical agents. With the progress of civilian surgery, surgeons became more complacent and infection had apparently been conquered.

World War I shook us from our smug satisfaction by presenting to a generation of surgeons, who thought wound infection a condition of the Dark Ages, a clinic in which all wounds suppurated. Accepted techniques and chemicals were suddenly powerless. There were at first many attempts to find new and more powerful antiseptics. The failure of these methods led to a realization that wound repair like growth comes from within and that the surgeon can only

*From the Department of Surgery, University of Michigan, Ann Arbor.

aid nature to limit and to destroy infection. Without enumerating all the steps by which this change in viewpoint came about, one can state that at the end of the war it was the object of the surgeon to convert the wound produced by the missiles of warfare to a clean one. This was accomplished by removing foreign bodies and dirt of the battlefield along with devitalized tissue. This operation, carried out in the stage of contamination, was called débridement. The historical aspects of this operative procedure, originating with Baron Larrey, need not be elaborated here. It was finally realized that the state of the patient himself played an important part in the end result. It became apparent that lost blood must be replaced, shock must be overcome, and malnutrition must be corrected. Wounds already infected were treated in the later period of that war by several methods, all of which gave good results. The French employed a modification of Ollier's methods developed by him in the Franco-Prussian War. The principles of these methods included free drainage, thick absorbent covering, immobilization, and infrequent dressings. Carrell and Dakin's treatment, with frequent irrigation of a wound with hypochlorite solution combined with meticulous dressings, had many advocates. In the British Army the use of hypertonic salt or magnesium sulfate solutions was popular and efficacious. Free drainage and rigid fixation of the part were fundamental to all methods.

In the years that have intervened, surgery has made great progress in the scientific study of factors that influence the processes of repair. Much has been learned concerning the bacteriology of infections and the use of chemotherapeutic drugs. An appreciation of the inhibitory effects of malnutrition has resulted in new concepts concerning the role of vitamins and hypoproteinemia in wound healing. Dr. Winnett Orr improved the Ollier technique and applied it successfully to the treatment of osteomyelitis and infected wounds of bones and soft parts. This method was used with remarkable results by Trueta in the Spanish Civil War. All these advances in our knowledge are applicable to wounds produced by any means and will be briefly reviewed.

The capacity for regeneration and repair as related to wound healing involves certain fundamental principles. To speak of the wounds of warfare categorically and to consider them apart from the traumatic and surgical wounds of civilian life may lead to certain omissions in the consideration of the general features of wound healing.* The influence of selective, purposeful mechanical pressure on wound healing has been discussed by Blair.[3] He has emphasized that pressure limits the amount of plastic substance that pours into the wound, prevents stasis, controls oozing, and eliminates dead spaces. Twyman[4] has demonstrated that epithelium proliferates readily when subjected to pressure that restrains the growth of connective tissue. For a rise in temperature of 10° C. the rate of cicatrization may be increased twofold.[5, 6] Other factors include the influence of electricity, radiant energy, diet and metabolism, vitamins, hormones, and the sympathetic nervous system.

---

*Quantitative factors may vary with the size of the wound and with wounds of different individuals and different species. Other well-known qualitative factors exert a potent force on the rate of healing. The accelerated healing observed in younger individuals indicates that healing in the young is more rapid than in adults because fibroplasia begins earlier.[1, 2]

The divers factors influencing such basic phenomena as fibroplasia, inflammation, and regeneration deserve more careful consideration than, perhaps, the empirical use of certain therapeutic drugs. Likewise, the reparative processes which follow wounds of mechanical, chemical, electrical, or thermal agencies do not need individual consideration, since there is little difference in the injuries inflicted.

Variations in terrain may influence the bacteriologic flora of the soil, and the increased mobility of the front presents problems in transportation which may result in delay in treatment. The indiscriminate mass attacks directed against densely populated cities, coupled with the increased effectiveness of air forces, have often resulted in a multiplicity of wounds and numbers of wounded out of proportion to existing facilities for their care. The increasing range of artillery fire, combined with the mobility of the front and the omnipresent airplane in turn, tends to limit the type and the amount of operating permissible at the front. Notwithstanding these difficulties, the general features of wounds remain the same from the physiologic standpoint, and those of warfare do not differ in any basic respect from those of civilian life.

WOUND INFECTION

Approximately 75 to 80 per cent of all wounds in modern warfare occur as a result of low velocity projectiles from artillery fire.* The type of wound commonly received is lacerated and contused, usually contaminated by foreign bodies, and requires extensive débridement to facilitate its healing and to avoid systemic infection. Unfortunately, it is true that many wounds escape contamination at the time of infliction only to become grossly contaminated before definitive treatment is completed.

(a) *Bacteriology.* If one divides the organisms introduced into the wound at the time of infliction from the organisms introduced at some later date, the former may be referred to as primary infection and the latter as secondary infection. It is significant that the organisms found in a primary infection are relatively nonpathogenic and grow poorly in unaltered blood or tissues. They will multiply, however, and produce clinical signs of infection when there is devitalization of tissue and when the antitryptic power is lessened by the breakdown of leucocytes. This is especially true of the spore-bearing anaerobic bacilli associated with gas gangrene and tetanus. These organisms are capable of producing infection and toxemia only when there has been alteration of blood and tissues. The hemolytic streptococcus is rarely present at this stage. The source of the primary infection is usually from the soil, the skin, or the clothing. The missile itself is frequently sterile. In the soil, in the air, and on clothing most of the bacteria are relatively nonpathogenic as compared to the hemolytic streptococcus.

*The value of physical prophylaxis of wounds is apparent from the fact that cranial wounds have been reduced from an incidence of 15 to 3 per cent since the introduction of the steel helmet. It is significant that the great majority of wounds are caused by small irregular missiles of low velocity. Although the armor necessary to protect against high velocity bullets may weigh as much as 20 pounds, compressed fiber or bakelite panels (with the same tensile strength as aluminum and one-half the weight) will suffice for protection against shrapnel and trench-mortar splinters. It is logically assumed that many chest and abdominal wounds, which in the past have been caused by low velocity missiles, may be prevented by the use of some form of lightweight body armor.

Secondary infection is of greater clinical import, these infecting bacteria reaching the wound through other agencies. Hemolytic streptococci are usually spread from patient to patient by the personnel caring for the wounded. In the last war it was shown that at the casualty clearing station only 15 per cent of the wounds exhibited the presence of the hemolytic streptococcus, but after a week at a base hospital over 90 per cent of wounds were infected with this organism. Droplet infection from the nose and throat, infected dust, instrument, and bed linens are important contributing factors. It has been shown that if the floor is treated with crude liquid paraffin or with certain proprietary preparations, subsequent sweeping of the floor does not cause large numbers of streptococci to appear in the air from infected dust.[7] In pre-Listerian days the spread of *B. pyocyaneus* through a surgical war was well known, since its presence was revealed by the characteristic color. The spread of *B. pyocyaneus* from patient to patient is not usually a matter of great moment to the patient, since this organism is of low virulence, but it is of the utmost importance as an illustration of some of the failures in technique in the care of wounds.

(b) *Operative Technique.* The success or failure of any débridement depends on the persistence in the wound of nonviable tissue or gross foreign matter. Although as early as 1897, a German surgeon, P. L. Friedrich, published experimental work emphasizing the treatment of contused wounds as if they were neoplasms, the factor of vitality of tissues as the decisive element in infection has not been given its due importance.[8] In the Franco-Prussian War only 9 per cent of the wounds were caused by shellfire and approximately 90 per cent by bullets.[9] With the development of modern warfare, the ratio has been almost completely reversed. Approximately 75 to 80 per cent of all wounds in modern warfare occur as a result of low velocity projectiles and, therefore, require débridement.

The ideal for which one must strive is to make a clean wound out of a dirty one and above all else not to make it any dirtier. Without appearing didactic one may enumerate sound surgical principles which have stood the test of time. Excessive trauma and rough handling of tissue with improper instruments may only serve to increase the amount of devitalized tissue, thereby defeating the primary purpose of the débridement operation. Failure to obliterate dead spaces and the mass ligation of tissue likewise contribute to the incidence of infection rather than help to prevent it. The indiscriminate use of irritating chemicals in the wound should be vituperatively condemned as an agency capable of increasing the devitalization through coagulation of protein substances in the cells.

Suture materials should be chosen with care. The reiteration of the theme that one type of suture will persistently give good results in the hands of anyone is to be condemned. Rather more emphasis should be placed on the effects of trauma, tension, incomplete hemostasis, and a consideration of various pathologic conditions. Well-controlled experiments[10] emphasize, however, that all sutures are foreign bodies and as such should be used sparingly. Foreign bodies when implanted into tissue normally cause a reaction characterized by leucocytic infiltration, the accumulation of serum and fibrin, increased heat, and

even swelling. A foreign body reaction superimposed upon attempts at repair in a healing wound will delay the onset of fibroplasia, and infection will be more frequent. Theoretically, then the ideal suture is one that causes the least reaction in tissues, thus allowing the normal prompt initiation of fibroplasia. When healing is complete, the suture should either disappear from the tissues, or if nonabsorbable, its continued presence should not prove objectionable.

Many objections raised in the past in regard to catgut pertain primarily to its large size. If the smaller sizes of chromic catgut are used with interrupted sutures, many objections which in the past have been reiterated will no longer be tenable. In this connection it is well to point out that there appears to be little indication for the use of plain catgut in any situation. The intensity of the leucocytic reaction incited by its presence is roughly twice as great as in the instance of chromic catgut, and in certain instances its holding strength is negligible in three or four days. The difference in cellular reaction between the absorbable and nonabsorbable sutures may in part be attributed to the large caliber of catgut which was frequently used. It is obvious that no reason exists for the use of suture material with a tensile strength in excess of the holding power of the tissue itself.

There is no great difference in cellular reaction when silk, cotton, steel wire, or nylon is used. However, cotton appears to possess certain advantages. Cotton has a natural twist which is maintained after implantation in tissue, and, therefore, there is less of a tendency for granulation tissue to infiltrate the interstices of the individual fibers to serve as a possible nidus of infection or as a source of a draining sinus. In addition, cotton being a vegetable fiber is chiefly cellulose whereas silk is mainly protein. In general, proteins incite more reaction than nonprotein substances. From an economic standpoint cotton possesses other advantages: a mile of cotton costs about 50 cents, while a similar length of catgut would cost somewhere around $200! The threatened impending shortage of silk need cause little concern among the many proponents of nonabsorbable sutures inasmuch as cotton appears to fulfill the criteria for an all-purpose suture. It does not delay the onset of fibroplasia, and its continued presence in the wound is not objectionable. Even in the presence of grossly contaminated wounds, the development of a draining sinus or extrusion of the sutures is almost never seen.

Stainless steel wire appears to be a suture material which is tolerated extraordinarily well by tissues. It is practically impossible to attribute any type of quantitative reaction to its presence in either experimental or clinical studies. Since 1937 it has been used for suturing the fascial layers in approximately 95 per cent of several thousand laparotomies at the University of Michigan with excellent results. It has never been necessary to remove them from an infected wound, and the incidence of infection itself has been substantially lowered by its use. Similar experience is related by Jones of the Cleveland Clinic, who reports a diminution in the incidence of infection in combined abdominoperineal wounds from 28 to 2 per cent directly attributed to the use of stainless steel wire in the abdominal wound.[11] It is easily sterilized, resists deterioration, and should lend itself well to the requirements of military surgery.

There are certain instances, however, where the use of catgut will definitely lead to inferior results. Nonabsorbable sutures are an integral part of standard techniques of tendon suture which have resulted in substantial improvement in this phase of surgery, as noted by many surgeons. O'Shea's study of 870 cases of severed tendons has shown that the use of catgut was followed by suppuration in 15 per cent, whereas when silk was used, the incidence was reduced to 3 per cent.[12-15]

(c) *Skin of Patient.* The adequate preparation of the skin before beginning débridement is just as important as in an operation for appendicitis. Utilization of "skin technique" and isolation of the wound margins with drapes will result in a diminution in the introduction of secondary invaders into the wound. That the skin is a potent source of contamination is apparent from the experiments of Carraway.[16] In clean laparotomy wounds bacteriologic studies made on the knife blade employed in the initial skin incision yielded positive cultures in 20 per cent of the instances.

(d) *Hands of Personnel.* The benefits accruing from vigorous and methodical scrub scarcely need mention. This one procedure, along with the wearing of sterile gloves, has probably contributed more to improvement in wound healing than in any other single factor. Wherever it is possible, aseptic technique should be rigorously preserved. Even slight compromises in technique are certain to jeopardize results.

(e) *Air of the Operating Room.* Bacterial dissemination in the air of the operating room is influenced by a wide variety of factors. The whispering and talking of traffic and galleries increase the bacterial count tremendously and emphasize the need for the wearing of masks by all who enter the operating theater. The care of the floors and walls where a large number of patients are being treated is important to prevent the spread of infecting agents. The good results obtained by the sterilization of air either by formaldehyde (Gudin[17]) or ultraviolet light (Gardner and Hart[18]) illustrate the importance of keeping the bacterial count of the air of the operating room at minimal levels.

(f) *Nose and Throat of Personnel.* At one time Meleney found that 30 per cent of his operating team harbored hemolytic streptococci in their *noses.* This serves to illustrate the necessity for covering the nose as well as the mouth with some type of serviceable nonpermeable mask. Many masks in common use are notoriously ineffective in this regard, since they allow free permeation of bacteria through the interstices of the material. Epidemics of upper respiratory infection among the operating personnel will frequently result in an increase in the incidence of wound infection unless the strictest precautions are observed. Meleney has shown that from 35,000 to 60,000 bacteria may fall upon the operating field in one hour.[19]

MANAGEMENT OF THE WOUND

Where these principles are observed, excellent results will be obtained. Only the most enthusiastic of idealists would deny that the conditions of warfare will frequently preclude adherence to these well-founded fundamentals. However, when compromises in technique must be granted, it is important to recognize them as such and to plan accordingly in the management of the wound. Sur-

geons of evacuation hospitals soon become specialists in débridement, but their experience should not be obtained at the expense of the early wounded. Successful débridement will be accomplished only through a sound knowledge of anatomy and the general features of wound healing. It must include excision in mass of all torn, crushed, discolored and noncontractile muscle, along with loose bodies and devitalized bone. Primary closure of these wounds may be undertaken only exceptionally, and it is here that judgment and experience are of prime importance.

Ogilvie,[20] in an excellent discussion on the treatment of wounds, carefully divides the progress of wound infection into two stages: contamination and infection.

In the first stage, contamination, bacteria which have been carried in by the inflicting agent lie on its surface, in bits of dirt, clothing, fragments, or in blood clot. This is the stage in which the bacteria can be removed by thorough mechanical cleansing.

In the second stage, infection, the bacteria profiting by the culture medium available have multiplied and started to invade the host. For practical purposes the stage of contamination may be considered as lasting eight hours before it passes into that of infection. Methods of closure are governed by the age of the wound, an estimation of the degree of contamination, and the general facilities at hand.

(*a*) *Primary Closure.* The state of the patient's skin and clothes and the appearance of the wound will aid in an estimation of the probable degree of infection. Doubt as to the condition of the wound should always incline one to pessimism rather than to mistaken optimism. Primary closure may be undertaken only exceptionally when the skin edges come together without tension, when the wound is seen very early, and when there is opportunity for continuous and careful observation of the patient subsequently. In addition, the contamination should be minimal and facilities for adherence to the strictest operative asepsis should be at hand.

Recent observations in actual battle conditions are as follows:

"A large proportion of wounds have been operated upon in the forward area. The recognized débridement was carried out in as many early cases as possible. The wounds were saucerized, sulfanilamide was dusted on the surface, followed by a vaseline dressing or loose pack. *All these cases did extremely well.* Some wounds had been sutured, but with the exception of a very few cases, *were badly septic on arrival at the base*, with sutures cutting out and in pain from suppuration under tension."

The same remarks are applicable to amputation. Those with complete skin closure have seldom met with success. They likewise have arrived at the base hospitals with the sutures cutting out, the skin sloughing, and with pus in the deeper tissues. Amputation under battle conditions should be planned, if possible, to permit of reamputation at one of the sites of election. Whenever possible, flaps should be cut and allowed to fall over the end of the stump.

(*b*) *Delayed Closure.* A delayed type of primary closure may occasionally be utilized in the wound that has gone beyond the rather arbitrary eight- to

twelve-hour limit.[21-24] A simplified technique for carrying out this procedure in dirty wounds has been described in a previous publication.[25] The skin sutures are placed at the time of the débridement but are not tied, and a pack left in the wound for a twenty-four-hour period will set up an inflammatory reaction in the subcutaneous layers. Clinical experience indicates that this inflammatory reaction enhances the ability of the wound to deal with the subsequent development of infection. Little, if any, disadvantage results from leaving the skin unsutured for two or three days. If the wound remains comparatively sterile, delayed primary suture is a means of diminishing the period of hospitalization with reduction of disability.

Secondary suture may likewise be employed in certain selected cases. A septic wound of the soft tissues in the course of about fourteen days is usually relatively clean if proper treatment has been carried out. By this time necrotic tissue has separated, and the granulation tissue has assumed a healthy appearance. Information as to the bacteriology of the wound may be obtained by suitable laboratory procedures.

It is probably advantageous to clean carefully the wound with continuous wet dressings for two or three days before performing secondary closure.

It is becoming more and more evident that the best dressing for any raw surface is skin. Even though the new skin is merely a temporary covering to be later replaced with another type of graft for functional or cosmetic reasons, its early use may avoid weeks of pain along with disability from scar tissue contracture. The healing of extensive raw surfaces by secondary scar formation should be avoided wherever possible.

Padgett and Hood,[26] of the University of Kansas, have perfected a calibrated mechanical device for cutting large sheets of skin at virtually any predetermined level. Whereas the Thiersch graft as cut with the hand knife may vary from 0.010 inch to 0.018 inch, the dermatome graft of Padgett may be cut in any range of thickness from 0.008 to 0.024 inch. A full thickness skin graft in an adult varies in thickness from 0.032 to 0.040 inch. The "three-quarter" thickness graft is cut at a level 75 to 90 per cent of the thickness of the skin. The degree of contraction of the base of a wound is proportional to the thinness of the overlying graft. In some areas a thin graft may contract as much as 60 per cent. Many objections raised in the past to split-thickness grafts are invalidated when this thicker type of graft is employed as cut with the dermatome. The ease with which it may be used makes virtually anyone proficient in this particular phase of surgery.

ROLE OF SULFONAMIDES IN WOUND HEALING

It has been estimated (Colebrook) that streptococci are responsible for at least 70 per cent of all deaths subsequent to wound infection. Only 15 per cent of wounds culture streptococci within twelve hours, but at the end of a week of hospitalization the incidence increases to about 90 per cent. Droplet infection from the nose and throat, infected dust, instruments, and bed linens are important contributing factors. It seems, therefore, that if sulfanilamide were present in the circulating blood at the time the organism reaches the tissues, a systemic

infection might be prevented. There is reason to believe that the sulfonamide group of compounds have a high value in combating and preventing infection in wounds. Their systemic use after the wound is infected and after operation in the stage of contamination as a prophylactic measure is well established.

Sulfanilamide as a topical application for wounds was first used in the Spanish Civil War. The results of such treatment, however, were lost when Barcelona was captured with disorganization of the entire medical service. Reports from France reveal that sulfanilamide was used in the French Army, applied directly to wounds as a prophylactic measure. It has, of course, been used subsequently in both civilian and military surgery, with some difference in opinion as to its general effects. Where the number of wounded is out of proportion to the facilities at hand for their care, it has been shown through recent military experience that the local use of the sulfonamides may prevent the transition of the stage of contamination to that of infection. In such an instance débridement may be utilized after the eight- or twelve-hour arbitrary dead line rather commonly employed in the past.

Jensen and his associates[27, 28] were the first in this country to publish observations on the topical use of sulfanilamide in wounds. They demonstrated "that sulfanilamide is more effective when implanted locally than when given systemically in preventing wound infection. Local sulfanilamide will not prevent infections in the presence of massive doses of contaminating organisms, although it does appear to lessen the severity of these infections.

"Devitalized tissue will inhibit the activity of locally implanted sulfanilamide. Foreign matter will protect the contaminating organisms against sulfanilamide." Chemotherapy is no substitute for careful surgical débridement, immobilization of the part, and skillful general aftercare. More carefully controlled studies are necessary to the final evaluation of locally applied sulfa chemicals in lengthening the time element when débridement can be done in contaminated wounds. There is no substitute for meticulous operative treatment done at the earliest possible time. In both the immediate and postoperative care of the wounded man, it is well established that the general nutrition of the patient is extremely important. Shock, hemorrhage, dehydration, and starvation must be treated and overcome. Especially important is hypoproteinemia that may result from malnutrition, hemorrhage, or from a septic course with much exudation. Proper diet, transfusion, plasma, and amino acid mixtures must be used to correct this nutritional abnormality.

It has been shown that a proper vitamin intake is essential to orderly wound healing. Vitamin A is related to epithelial cell growth; vitamin B complex apparently influences proliferation of fibroblasts; vitamin C plays an important part in collagen formation and in fibroblastic growth. A food intake rich in these essentials is necessary for tissue repair.

Much remains to be learned from the observations and tests of experience, but we now face the care of those suffering tissue injury with more assurance. The scientific studies of the past two decades have given us a sound foundation on which to build new and better techniques.

REFERENCES

1. Lecomte du Noüy, P. E.: Cicatrization of Wounds, J. Exper. Med. 24: 451, 1916.
2. Lecomte du Noüy, P. E.: Une mesure de l'activité physiologique, Compt. rend. Soc. de biol. 109: 1227, 1932.
3. Blair, V. P.: The Influence of Mechanical Pressure on Wound Healing, Illinois M. J. 46: 249, 1924.
4. Twyman, E. D.: Epithelial Proliferation. Differential Pressure as an Aid in the Growth of Epithelium on a Denuded Surface, J. Missouri M. A. 19: 257, 1922.
5. Ebeling, A. H.: Cicatrization of Wounds, J. Exper. Med. 35: 657, 1922.
6. Ebeling, A. H.: A 10 Year Old Strain of Fibroblasts, J. Exper. Med. 35: 755, 1922.
7. Fleming, A.: Bacteriological Examination of Wounds, in Bailey, H.: Surgery of Modern Warfare, Edinburgh, 1941, E. & S. Livingstone, pp. 141-149.
8. Trueta, J.: The Treatment of War Fractures by the Closed Method, Proc. Roy. Soc. Med. 33: 65, 1939.
9. Lyle, H. H. M.: The Principles of the Surgery, Hospitalization, and the Evacuation of the Wounded in the Meuse-Argonne Offensive, Mil. Surgeon 84: 580, 1939.
10. Farris, J. M.: Tissue Reactions to Suture Materials, Ann. Surg. 114: 159, 1941.
11. Jones, T. E.: Complications of One Stage Abdominoperineal Resection of Rectum, J. A. M. A. 120: 104, 1942.
12. Bunnell, Sterling: Repair of Nerves and Tendons of the Hand, J. Bone & Joint Surg. 10: 1, 1928.
13. Bunnell, Sterling: Primary Repair of Severed Tendons. The Use of Stainless Steel Wire, Am. J. Surg. 47: 502, 1940.
14. Mayer, Leo: Repair of Severed Tendons, Am. J. Surg. 42: 714, 1938.
15. O'Shea, Maurice C.: The Treatment and Results of 870 Severed Tendons and 57 Severed Nerves of the Hand and Forearm, Am. J. Surg. 43: 346, 1939.
16. Carraway, C. N.: Some Causes of Occasional Infections in Clean Abdominal Cases, South. M. J. 22: 554, 1929.
17. Gudin: Asepsie fictive et sterilisation totale, Presse méd. 44: 355, 1936.
18. Gardner, C. E., and Hart, Deryl: Recent Advances in Surgery from a Bacteriological Viewpoint, Surgery 1: 458, 1937.
19. Meleney, F. L., and Stevens, F. A.: Postoperative Haemolytic Streptococcus Wound Infections and Their Relation to Haemolytic Streptococcus Carriers Among the Operating Personnel, Surg., Gynec., & Obst. 43: 338, 1926.
20. Ogilvie, W. H.: Treatment of Wounds, Brit. M. J. 1: 132, 183, 1938.
21. Buxton, S. J. D.: Injuries of the Lower Limb: I., Brit. M. J. 1: 1241, 1939.
22. Lauwers, E.: Traitement des plaies cranio-cerebralis par projectiles, Presse méd. 48: 30, 1940.
23. Leriche, R.: Apres l'exision des plaies musculaires par projectiles de guerre, suture primitive, platre ou suture retardee, Presse méd. 48: 1, 1940.
24. Weddell, J. M.: The Treatment of Wounds in War, Brit. M. J. 1: 785, 1939.
25. Coller, F. A., and Valk, W. L.: The Delayed Closure of Contaminated Wounds, Ann. Surg. 112: 256, 1940.
26. Padgett, E. C.: Skin Grafting, Springfield, Ill., 1942, C. C Thomas.
27. Jensen, N. K., and Nelson, M. C.: Local Sulfanilamide in Compound Fractures, Surg., Gynec. & Obst. 75: 34, 1942.
28. Jensen, N. K., Johnsrud, L. W., and Nelson, M. C.: The Local Implantation of Sulfanilamide in Compound Fractures, Surgery 6: 1, 1939.

HEALING OF WOUNDS*

Lieut. Col. Warner F. Bowers, M.C., U. S. Army

Wound healing is a process which intimately concerns every surgeon, but this fundamental subject has received only superficial consideration, for the most part, until very recently when the basic principles have begun to be more widely appreciated. This paper attempts to epitomize the known facts from various angles, basic and clinical.

BIOPHYSICS OF WOUND HEALING

As soon as a wound to tissue is sustained, serum and blood accumulate and coagulate in the wound space and to a lesser degree in the interstices between the surrounding uninjured cells. Into this damaged area, cellular elements of the blood, wandering tissue cells and new capillary loops are attracted. These capillary loops make up granulation tissue which pulls the wound edges together as the fibroblasts mature. This briefly is the entire process of normal healing and it is dependent on certain fundamental phenomena, namely: ameboid movement of cells, mitotic proliferation of cells and maturation of cells engaged in fusion of wound surfaces. Ameboid movement of cells is stimulated by the presence of blood, tissue fluids and a large number of other substances. Inadequacy of oxygen and nutritive substances in the relatively ischemic border of the wound may cause tissue hunger and anoxia followed by cell division and ameboid activity of fibroblasts and endothelial buds. Immediately after tissue injury the lytic phase of wound healing begins and here the dead tissue elements are removed by phagocytes. This also is called the lag phase because reparative processes do not begin until toward the end of the lytic phase. Phagocytosis is followed by an influx of wandering connective tissue cells and then proliferation of the invading fibroblasts begins. Now, two fundamental processes begin to operate. The stereotropic response of growing cells to surfaces causes the growing fibroblasts to elongate along the fibrils of the fibrin network in the coagulum, filling the wound space just as epithelial cells show ameboid movement along plane surfaces of granulation tissue and beneath crusts. This stereotropic response thus causes the fibroblasts to grow out and invade the wound space on the trellis of fibrin fibrils. A second factor which operates toward the same end is that of centrifugal force which directs growing cells away from their own kind and so into the plasma mass in the wound space. Endothelial buds also exhibit this phenomenon which may be an electrochemical mechanism; like cells with like surface charges tending to repel each other. Wounds become firm as the maturing fibroblasts contract and surface epithelialization progresses. The expected time of healing of any wound readily can be computed mathematically

*Chief of Surgical Service, Station Hospital, Camp Gruber, Oklahoma.
A lecture delivered before the Omaha Medical Troop School.
Reprinted from The Military Surgeon 90: 140 (February), 1942.

using the Carrell-DuNouy[1] formula which is calculated on the basis of surface area. These computations also apply to the healing of granulating cavities such as those seen in empyema. I have previously shown this to be the case in some unpublished work quoted by Carlson.[2]

SYSTEMIC CONDITIONS INFLUENCING WOUND HEALING

1. Tissue age, whether adolescent, normal adult, senescent, or degenerated, influences the rate of wound healing not only because young tissues heal most readily but because old tissues are apt to show vascular changes which retard healing. 2. The state of hydration of the tissues as determined by water, electrolyte and protein balance in the blood exerts a profound effect on wound healing, and these factors will be discussed separately and in more detail later. 3. Normal nutritional balance is a somewhat vague term which probably covers the aggregate of many of the other factors which influence wound healing. 4. Vitamin balance will be discussed in detail later and will be found to be one of the most fundamental but least known influences on wound healing. 5. The general state of circulation and blood picture influence wound healing as they affect the interchange of cellular and ionizable elements between normal and damaged tissues.

LOCAL FACTORS INFLUENCING WOUND HEALING

1. There is a quantitative relationship between the amount of killed or damaged tissue in the wound and the time required for phagocytosis and repair. 2. Vascularity of the tissues involved is of prime importance in wound healing. Avascular tissues must depend on lymph spaces for interchange of nutritive and waste materials, and since most authorities deny that lymph flows, this interchange depends upon diffusion gradients for exchange of ionizable substances and ameboid motion for ingress of cells. 3. As a corollary to the preceding statement, the local blood supply to the wound must be intact, for no matter how vascular the tissue, if the blood supply is jeopardized by trauma, healing will be retarded in direct ratio. 4. The amount and character of the exudate in the wound space and in the tissues bordering on the wound determine the rate of healing in large measure. 5. The number and nature of the infectious organisms in the wound space and bordering tissues have a profound bearing on wound healing as infection throws an added burden on the reparative mechanism. 6. The number and type of foreign bodies which must be encapsulated or extruded from the wound influence the rapidity of wound healing.

RELATION OF TISSUE pH TO WOUND HEALING

Normal tissues preserve a neutral reaction by means of diffusion between the blood stream and local tissue spaces. Injured tissue accumulates acid metabolites and the breakdown of dead tissue also liberates split protein products which are acid in reaction. These factors influence the injured area and cause a shift in pH toward the acid side. Menkin[3] shows that neutrophilic leucocytes are attracted to slightly acid areas, and these cells therefore congregate in the injured area. If acidity exceeds the optimum point, the neutrophilic leucocytes are killed and macrophages are attracted. If the macrophages are able to phagocytose enough dead tissue to decrease the acidity, the inflammatory

reaction regresses; but, on the other hand, if the acidity increases, the macrophages also are killed and suppuration results. As the neutral stage is regained, such cells as the lymphocytes and plasma cells migrate to the site of reaction. From this discussion it seems that chemotaxis is largely responsible for the accumulation of the various cellular elements at the site of inflammatory reaction.

ROLE OF VITAMINS IN WOUND HEALING

Vitamin A deficiency causes such clinical manifestations as night blindness, photophobia, dry skin, dry conjunctivae, blepharitis, follicular hyperkeratosis, and numerous or severe infections. This latter may be of some importance in wound healing but such terms as "local tissue immunity" and "site of local lowered tissue resistance" usually are employed only to cover ignorance. About 35 to 50 per cent of people show deficiency in Vitamin A by the photometric test; however, this test is not generally accepted as accurate. About 12 per cent of individuals show clinical deficiency of vitamin A, as evidenced by one or more of the previously mentioned clinical manifestations.

Of the vitamin B complex, B_1 or thiamin chloride seems to be the only factor of importance in the healing process. B_1 seems to be identical with one of the components of the carboxylase enzyme system governing metabolism in nerve tissue. Therefore vitamin B_1 is of importance in the healing of injuries to nerve tissue.

Vitamin C (ascorbic or cevitamic acid) is the most essential vitamin in the healing process because intercellular substance in general and in the capillary bed in particular, as well as the collagen of all fibrous tissue, requires cevitamic acid for its production and maintenance. Lack of vitamin C causes hemorrhage into the wound space with a lag in healing and a tendency to wound disruption. Vitamin C deficiency in experimental animals has been shown to delay return of tensile strength in wounds. About 40 per cent of indigent clinic patients are deficient in vitamin C and all patients over seventy years of age show a relative deficiency. Patients placed on a strict ulcer diet become deficient in vitamin C within four days. These figures are arrived at by using the skin test or the capillary fragility test. Many superficial studies of wound dehiscence have been published and various factors have been blamed, but it seems reasonable and probable that vitamin C deficiency is the most universal and important factor. Vitamin C deficiency has many other interesting implications as has been emphasized recently by Levine,[4] who states that German soldiers receive 100 mg. of crystalline vitamin C daily.

Vitamin D is irradiated ergosterol which is related to cholesterol, one of the group of sterols which are of extreme importance in basic cell physiology because the sterols are responsible for the selective permeability of cell membranes. The presence of these sterols in the surface film enables the cell membrane to change its colloidal state from a water in oil to an oil in water emulsoid and, therefore, both oily and aqueous substances may be taken into the cell selectively.

Vitamin K, a water-soluble naphthohydroquinone, is responsible for maintaining the level of blood prothrombin. K is absorbed from the gastrointestinal tract only in the presence of bile so that in cases of biliary obstruction, K is not

absorbed, the prothrombin level drops and the patient tends to bleed. Vitamin K deficiency is of importance in wound healing only in patients having biliary obstruction.

The story of vitamins is not yet told but this seems to sum up our present knowledge of vitamins as they relate to wound healing.

RELATION OF STATE OF HYDRATION TO WOUND HEALING

Water is necessary to carry nutritive elements and waste products in solution and since these solutions must be isotonic with blood, relatively large quantities of fluid are essential. The many other implications of this important subject have been covered by the papers of Coller and Maddock.[5] Suffice it to say here that either too much or too little fluid retards wound healing. The state of hydration of the surgical patient may be gauged by: 1. The urinary output. 2. Specific gravity of the blood as measured by the falling drop method.[6] 3. The McClure-Aldrich salt edema test.[7] The surgical patient must have a daily urinary output of at least 600 c.c. and preferably 1,000 c.c. The falling drop test measures the concentration of the blood, and by the use of a simple chart blood protein levels may be quickly read off. The McClure-Aldrich salt edema test depends on absorption of skin wheals of normal saline solution, and the rapidity of absorption is an index of the tissue hunger for fluid and salt.

RELATION OF ELECTROLYTE BALANCE TO WOUND HEALING

Sodium chloride is the most important electrolyte and is almost entirely responsible for the osmotic pressure of intercellular fluid. Accumulation of salt in the tissues attracts fluid, edema develops, and edematous tissues do not heal properly because of altered pressure relationships and insufficient ingress and egress of materials. Decreased concentration of salt in the tissues causes a loss of fluid into the vessels with consequent tissue dehydration. Adequate fluid balance, therefore, depends on salt balance, and both are very important in wound healing.

RELATION OF LEVEL OF PROTEINEMIA TO WOUND HEALING

Protein is the most important colloid element of blood, and as the quantitative level of blood protein is decreased, the osmotic pressure and diffusion gradient are so altered that fluid tends to leave the vessels and accumulate in the tissue spaces. Blood protein levels of lower than 5 mg. per cent result in this type of edema. Deficiency of protein as well as edema retards wound healing. Ravdin[8] has shown that abdominal wound disruption developed in over 70 per cent of dogs operated upon in the presence of hypoproteinemia. There was little or no evidence of fibroplasia in these cases up to two weeks after operation. On the other hand, a high protein diet speeds up wound healing and almost eliminates the lag phase.

RELATION OF METABOLIC RATE TO WOUND HEALING

Theoretically, an increase in basal metabolic rate should speed the healing process, but such does not seem to be the case. Goiter incisions in patients with very high metabolic rates do not heal any more readily than other types of

incisions about the neck. Routine administration of thyroid extract postoperatively to speed wound healing and increase the circulatory rate to prevent thrombophlebitis has been advocated but has not proved to be of value.

RELATION OF LOCAL BLOOD FLOW TO WOUND HEALING

Local blood flow brings in most of the cellular elements which are so important in wound healing, brings in nutritive elements, and carries off metabolites. In addition, there is a very important mechanical factor to be considered. If a large vein is damaged, it should be ligated because the thin wall precludes suture, and since veins usually are multiple, sufficient venous channels will remain. If a large artery is damaged, it should be sutured if feasible but if this is impossible, a very different physiological problem arises. Blalock has shown that if a large artery is ligated, its accompanying vein also should be ligated. This procedure has the effect of causing blood to remain longer in the involved area and thus the injured tissues have more time to make use of it. If the artery alone is ligated, the venous channels of return overbalance the arterial supply and the area is drained of blood. Ischemic gangrene may be the result. This is a very difficult point to make, and while many surgeons advocate and teach it, few have the courage of their convictions. The rationale is clear and I have seen it work in practice.

EFFECTS OF TEMPERATURE ON WOUND HEALING

The application of heat to most potentially infected wounds by the use of hot packs, hot water bottles or light cradles is almost universal but may be very dangerous. There may be three serious disadvantages to the use of heat on wounds. 1. Heat may speed up local tissue metabolism to the point where the damaged vessels cannot adequately supply the increased demand for fuel and gangrene results. This is more frequent than is realized in such cases as diabetic infections which progress to gangrene. 2. Heat cannot be carried away because of damaged vessels so that a cumulative effect is present and causes increased tissue damage. 3. Heat augments capillary filtration and so increases local edema which may or may not be beneficial as will be discussed later. It is a safe rule never to use external heat of more than 100 degrees, and a thermometer always should be hung in the light cradle. Refrigeration in the presence of vascular damage probably is more rational than the use of heat. Refrigeration decreases metabolic needs so that a damaged circulation may be adequate. Fay has applied this principle to the treatment of carcinoma because malignant tissue has a higher metabolic rate than normal tissue, and refrigeration may cause death of tumor tissue before normal tissue is affected.

ROLE OF PRESSURE AND TISSUE TENSION IN WOUND HEALING

I previously have shown[9] that in a hollow viscus distention of the lumen or increase in the tissue tension will cut off blood flow sufficiently to cause inflammatory changes within the walls and this will progress to gangrene and perforation even in the complete absence of bacterial infection. Wound healing in other tissues is slowed by increased pressure or tissue tension because of reduction in blood flow or hindrance to physicochemical processes. Brooks[10]

showed that the period of viability of tissues rendered completely anemic by pressure is remarkably constant at 18 hours. The time element seems to be much more important than the degree of pressure because very low pressures may cause gangrene if applied constantly over a long period of time. Edema or external appliances may exert sufficient pressure to cause tissue damage and mild degrees of damage heal by fibrosis which, in such cases as Volkman's contracture, may lead to serious dysfunction.

IMMOBILIZATION AND ELEVATION IN THE TREATMENT OF WOUNDS

Immobilization is a well-established principle in the treatment of fractures, but its benefits in soft tissue wounds are not so generally appreciated. Complete immobilization in soft tissue injuries not only facilitates healing but also relieves pain, prevents deforming contractures and facilitates nursing care. Immobilization for soft tissue wounds was used extensively in the late Spanish War and has been exhaustively discussed by Trueta and others.

Elevation of the injured part makes use of the force of gravity to aid in return of blood and lymph from the site of injury and is an extremely important mechanical principle. The fundamental question as to whether the inflammatory reaction to healing and infection is the result of bacterial action or is a defense mechanism on the part of the host has never been satisfactorily answered. If the former, then treatment should combat the reaction, and if the latter, therapeutic measures should augment the reaction. Edema fluid is said to contain antibodies, and swelling may occlude tissue spaces and inhibit bacterial spread. On the other hand, edema increases pain, decreases circulation, slows healing, and may provide a fluid medium through which bacteria disseminate. Elevation of the affected part definitely reduces inflammatory edema and makes the patient more comfortable. Healing is facilitated but there are two dangers connected with the use of elevation. 1. Elevation of the lower extremity puts an extra load on the vascular system because blood does not flow up hill without work. Particularly in older patients the angle of circulatory efficiency must be watched closely. The toes of elevated extremities should be checked repeatedly for drop in temperature, and the vessels must be palpated for adequate circulation. I have seen a number of cases of gangrene develop from excessive elevation of the leg in young persons. 2. Elevation causes pus to settle by gravity and travel along fascial planes toward the trunk. I have seen several huge gravitational abscesses develop under casts applied with the leg in elevation where the abscess was some distance proximal to the original infection. The papers of Wangensteen[11] discuss the factors of immobilization and elevation in the treatment of wounds in considerable detail.

PRIMARY VS. DELAYED CLOSURE OF WOUNDS

The decision as to whether primary or delayed closure of a wound is to be employed depends on the time that has elapsed since the injury and also on the degree of obvious contamination. Primary closure in a clean wound is indicated up to 8 hours after injury, but recently I have seen wounds of much longer duration, involving tendon suture, primarily closed with good results by implanting

sulfanilamide powder at operation. Ordinarily, however, dirty wounds or clean wounds seen after 8 hours are treated by delayed closure. If the wound meets the criteria for primary closure, it should be flushed with large quantities of saline solution or washed repeatedly with soap and water. Bad habits are difficult to eradicate so that the instillation of so-called antiseptic solutions into wounds still is practiced widely. Closure should be accurate but without tension. Silk sutures may be used in clean cases, but catgut is safer because of the disagreeable sequelae if silk is used in the presence of infection. Catgut has a number of disadvantages, among which may be mentioned the fact that it is a foreign protein and occasions a certain amount of inflammatory reaction during its ultimate liquefaction and absorption. Certain patients absorb catgut very rapidly, and there is some question of allergy in these cases. In wounds which are doubtfully clean, it is possible to place adhesive tape close along the wound edges and then to suture the adhesive instead of the tissue. This procedure makes it a simple matter to open the incision if infection develops. If the wound does not meet the requirements for primary closure, it should be treated in one of the ways to be discussed later and then, when a smear from the granulating surface shows a bacterial count of less than 10 organisms per high power field, secondary closure may be done or the wound may be covered by a skin graft.

DÉBRIDEMENT OF WOUNDS

Contused and multiple lacerated wounds require careful excision of all obviously devitalized tissue because of the danger of infection, particularly by the anaerobic organisms of tetanus and gas gangrene if dead tissue remains to use up oxygen and act as a culture medium. A study of ballistics shows that for various sizes and velocities of projectiles, there are definite areas of tissue on all sides of the injury which subsequently will die as a result of the concussion. Therefore, it is important for the surgeon to know what type of projectile caused the injury in order to determine how much tissue to remove. Poor judgment in this matter may seriously affect subsequent healing.

ANTISEPTICS IN INFECTION AND WOUND HEALING

Many kinds of saprophytic bacteria and higher organisms live on the normal human skin, the number and kind depending on the environment and the degree of personal hygiene of the individual. A clean skin usually shows 12 staphylococci or diphtheroids per square centimeter. Other common organisms in addition to fungi are spore formers, the colon group, and Bacillus proteus. This indicates that all wounds are at least potentially contaminated but simple flushing of wounds with saline solution or soap and water usually suffices to remove these nonpathogenic organisms. However, from time immemorial men have searched for the magic colored solution which will kill bacteria *but not* harm tissues. Notwithstanding the expensive and varicolored solutions *on* the market today, such a desideratum has not yet been attained. The practice of pouring these solutions into defenseless wounds simply increases the tissue burden and should be discontinued. Novak[12] showed that all common alcoholic antiseptics kill 96 to 99 per cent of skin organisms, but 50 per cent alcohol plus 10 per cent acetone alone kills 97 per cent. It, therefore, seems irrational to pay

\$10 a gallon for certain commercial solutions. Novak made up a solution of 0.5 per cent cresol and 0.07 per cent bichloride of mercury in 50 per cent alcohol and 10 per cent acetone solution. This solution kills 100 per cent of diphtheroids, 99.6 per cent of staphylococci, costs but 60 cents a gallon, and has been successfully used clinically for at least three years at the University of Minnesota General Hospital. Novak solution has been used for skin preparation at the Station Hospital, Ft. Leonard Wood, Mo., since the hospital opened on May 3, 1941, with an extremely low incidence of wound infections.

TREATMENT OF INFECTED WOUNDS

The fact that such a large number of agents have been recommended for the treatment of infected wounds indicates that the ideal method has not been discovered. However, it is well to bear in mind the outstanding points in the use of these various substances.

Dakinization. Dakin's solution is an aqueous buffered solution of sodium hypochlorite which liberates chlorine. Chlorine has the effect of liquefying fibrin, dissolving dead tissue, killing bacteria, and, therefore, is beneficial in any infected wound. The solution has the disadvantages of rapid deterioration, short action, objectionable odor and requires an elaborate system of bottles and tubes for proper use. Dakin's solution dissolves the cornified layers of normal skin and, therefore, the area around the wound must be protected by vaseline gauze. The solution will sterilize a wound in 48 hours if correctly used and will transform pus into a transparent, mucinous substance with only a slight choline odor. Azochloramide is a more stable preparation which liberates chlorine over a longer period and consequently is less irritating to normal tissue. Dichloramine T is an oily preparation which disintegrates still more slowly. Dakin's solution was extensively used in the last World War, but it is predicted that it will be little used in the present war.

Maggot Treatment. Maggots were first introduced in the treatment of chronic osteomyelitis because it was observed that infested cases were found to be the cleanest. The application later was broadened to include all infected necrotic wounds. There are several ideas as to why the larvae are effective. 1. It has been suggested that the larvae actually eat the dead tissue and purulent exudate, thus mechanically cleaning up the wound. 2. It has been thought that larval excretions are responsible for the healing effect. 3. Dead larvae are thought to liberate a substance which is bactericidal. The use of live maggots is objectionable to patients because of the crawling of the maggots and from the esthetic viewpoint. Furthermore, the treatment is difficult to carry out because it is hard to obtain sterile maggots as needed. The problem has been approached by an effort to isolate the effective principle and by the application of macerated larvae in saline solution or ointment form.[13] Experimental evidence shows that these extracts and ointments definitely promote healing, and at present it does not seem necessary to use live maggots.

Cod Liver Oil. Cod liver oil is very valuable in the treatment of any infected wound and in prophylaxis in any open wound. The oil discourages bacterial growth and has a marked stimulating effect on granulation tissue. There

may be some truth in the claim that vitamin D is the effective agent because it is known that oxidized odorous oil is more efficacious than the highly purified product. Also, irradiation of other oils increases their healing properties. Cod liver oil may be used to fill cavities and soak dressings or may be used as a 30 to 50 per cent ointment in vaseline. Cod liver oil is of great usefulness in the treatment of infected wounds, empyema, chronic osteomyelitis, suppurative arthritis, burns, arteriosclerotic and diabetic gangrene.

Oxidizing Agents. Certain bacteria grow best in an atmosphere of lowered oxygen tension and grow well in symbiosis because one organism uses up oxygen, thus making an anaerobic medium for the other. A microaerophilic streptococcus is such an organism and causes a gangrenous spreading infection. One means of treatment is débridement followed by the application of a substance which breaks down to liberate oxygen. Meleny has popularized the use of zinc peroxide in such cases. This agent comes in powder form which is sterilized and then made into a thin paste with sterile water. The wound then is filled with paste-saturated sponges and a large airtight dressing is applied to inhibit evaporation and to prevent the rapid escape of oxygen.

Chlorophyll. Various vegetable substances recently have been advocated for use in treatment of infected wounds. Of these, chlorophyll[14] has received the widest trial. Chlorophyll inhibits bacterial growth apparently by preventing bacterial enzymic digestion of cell membranes. It is used in aqueous solution or in an oily base for suppositories or ointments. It may be given intravenously, by mouth, or it may be used as an irrigating fluid. Chlorophyll cleans up the wound and stimulates granulation tissue. It has had extensive clinical trial in such cases as wound infections, empyema, fistulas, deep abscesses, acute infections of the nose, throat and ear, male and female urinary tract infections. This substance should be cheap, easy to prepare, and almost universally present. It is interesting to speculate as to what role the vitamins in green vegetation may play in the healing effect of aqueous extracts.

Barberry. Folklore remedies which have stood the test of time deserve scientific investigation in order to isolate whatever specific principle may be present. One example of such work is the recent investigation of barberry by Dick.[15] Barberry has been widely used for centuries by aborigines as an infusion to cure surface infections and gonorrhea. Experimental work shows the effective agent to be berberine, which in 1 per cent solution kills erysipelas streptococci in 8 hours. Clinically, berberine has been successfully used in wound infections, decubitus, and varicose ulcers and mouth infections. It is dangerous when administered intravenously.

Pectin. Another folk remedy of proved worth is the apple diet for infantile diarrhea. The effective agent now is known to be pectin, which is found to be bacteriostatic in aqueous solution and stimulating to granulation tissue. Tompkins[16] and others have shown its worth in the treatment of various wound infections.

Silicon. The use of silicon in the treatment of wounds has a very interesting background. Mountain dwellers of Virginia have a tradition that eating sand will cause peptic ulcers to cease bleeding, and this led to an investigation of

silicon. It is known that silicon in the lung causes intense fibrosis of the pulmonary tissue, and Gardner has shown that this is in inverse ratio to the size of the particles of silicon. It seems established then that silicon promotes fibrosis, and the idea that silicon might cause wounds to fibrose was given a clinical trial with striking results. Silicon was ground to the finest powder and used in all types of wounds and ulcers with prompt healing. Silicon was given by mouth for bleeding ulcers and by cystoscope for bleeding tumors with equally good results. I saw these cases demonstrated in the Department of Surgery at the University of Cincinnati in 1934 and was deeply impressed but to my knowledge the work never was published. They were able to show elevation in the level of blood silicon by oral administration with favorable results in wound healing.

Specific Sera and Antitoxins. Gas gangrene, caused by Cl. welchii, is a frequent complication of war wounds because these anaerobic organisms are almost universally found in dirt. This complication is much dreaded because the gas generated by the organisms rapidly spreads along tissue planes and produces pressure necrosis of tissue. Manson has shown a 35 per cent incidence of Cl. welchii in indolent leg ulcers, and this has given undue alarm to many surgeons doing amputations in cases of diabetic or arteriosclerotic gangrene. In a published series, Kennedy and I[17] have shown that 38 per cent of milk cultures made at operation and at the site of amputation were positive for gas-forming organisms, but in no case was antigas serum given and in no case did gas gangrene develop. This illustrates our point that most of these gas formers are relatively nonpathogenic, and if amputation is done according to the criteria we laid down, favorable results will follow in a higher than usual per cent of cases. Formerly, gas gangrene was treated by massive doses of antitoxin, but its value is open to question, and the danger of foreign protein sensitization is great. Injection of oxygen into the tissues has been used with some success, but the most beneficial treatment seems to be with the x-ray or the local use of sulfanilamide. Tetanus is caused by Bacillus tetani, also a dirt organism, and is the cause of a high mortality rate. There is nothing new in the treatment of this disease, but it is suggested that zinc peroxide, x-ray therapy, and sulfonamide derivatives give enough promise to warrant investigative work. One answer to the problem may lie in the universal toxoid immunization to tetanus now employed by the Army, but some alarming anaphylactic reactions are being reported.

X-ray Therapy. Since the striking work of Kelley in the treatment of gas gangrene, peritonitis, and other severe infections by x-ray therapy, it has been suggested that it be used in the early treatment of war wounds. Keating and Davis[18] suggest that portable x-ray therapy units be placed with mobile surgical hospitals which already have power plants mounted on trucks. All dirty wounds would be treated within four hours after injury. This procedure is not to be recommended for three reasons: 1. Slight overdosage of x-ray therapy delays wound healing and may cause other well-known and more serious consequences. X-ray therapy given in all surgical hospitals would require many more trained radiologists than are available. 2. Not only is there danger of overdosage at one sitting but it is quite likely that in the rush of battle evacuation, records might not be completed so that patients might be treated twice. Such mistakes are not entirely beyond the bounds of possibility. 3. Local implantation of sulfanilamide

is so astonishingly successful in combating wound infection that it should not be discarded in favor of x-ray therapy, and most authorities state that the two modes of treatment should not be employed in the same case.

Sulfonamide Drugs. Sulfanilamide and its derivatives seem to act as bacteriostatics by virtue of their power to prevent bacterial cells from utilizing oxygen. Sulfapyridine was introduced to combat staphylococci, but it is relatively insoluble, gives a higher incidence of gastrointestinal upsets, and now is little used. Sulfathiazole is well tolerated by mouth, in solution in cod liver oil, or in ointment form. Excellent results have been reported from its use in carbuncles, operative wound infections, decubitus ulcers, corneal ulcers, chronic osteomyelitis, empyema and subcutaneous abscesses.[19] However, sulfathiazole is less soluble than the original sulfanilamide which is preferred for local implantation.

Local implantation of sulfanilamide crystals was first used in compound fractures by Jensen,[20] who reduced the previous incidence of severe infections from 25 per cent to nearly zero. Implantation gives a local concentration up to 800 mg. per cent as compared with 10 mg. per cent which is attained with difficulty by oral administration of the drug. This concentration is so great that all organisms are affected, regardless of whether or not sulfanilamide is specific for them. Saturated solutions of the drug may be used to irrigate infected cavities with excellent results. Local implantation of sulfanilamide around complicated gastrointestinal anastomoses[21] has greatly reduced the mortality, and its use free in the peritoneal cavity in cases of peritonitis has been beneficial.

It is to be remembered, however, that sulfanilamide is absorbed with great rapidity from the peritoneal cavity, its maximum blood level often being reached in 15 minutes after implantation. For this reason, the liver may be fatally damaged unless care is taken to use no more than 5 grams of the drug. I have seen three patients die of toxic hepatitis following the intraperitoneal introduction of 10 grams of the drug. It is suggested that every soldier be furnished with 5 gram ampoules of sulfanilamide with instructions to break one and pour the contents into a wound, whatever the site, as soon as incurred. The question of having the soldier begin oral administration of the drug or one of its derivatives also, immediately after injury, should receive earnest consideration.

SUMMARY

Biophysical, mechanical, chemical and bacterial factors are briefly considered as they relate to wound healing. The use of various and diverse substances in the treatment of infected wounds is discussed and numerous points are raised with the hope that additional investigative work will be stimulated.

REFERENCES

1. Carrel, A., Hartmann, A., and DuNouy, L.: Healing of Wounds, J. Exper. Med. 24: 451, 1916.
2. Carlson, H. A.: Acute Empyema Thoracis, J. Thoracic Surg. 5: 393, 1936.
3. Menkin, V.: Inflammation, Arch. Path. 27: 115, 1939.
4. Levine, V. E.: Scurvy, the Soldier's Calamity, The Military Surgeon 89: 140, 1941.
5. Coller, F. A., and Maddock, W. G.: Water and Electrolyte Balance, Surg., Gynec. & Obst. 70: 340, 1940.

6. Foote, M. N., and Gerst, G. R.: Practical Application of the Falling Drop Method for Determining Surgical Prognosis, Am. J. Surg. 50: 316, 1940.
7. Hopps, H. C., and Christopher, F.: The McClure-Aldrich Test in Water Balance Following Operation, Surg., Gynec. & Obst. 69: 637, 1939.
8. Ravdin, I. S.: Hypoproteinemia and Its Relation to Surgical Problems, Ann. Surg. 112: 576, 1940.
9. Bowers, W. F.: Role of Distention in the Genesis of Acute Inflammation of Hollow Viscera, Am. J. M. Sc. 194: 205, 1937.
10. Brooks, B., and Duncan, G. W.: Effects of Pressure on Tissues, Arch. Surg. 40: 696, 1940.
11. Wangensteen, O. H.: Importance of Immobilization and Posture in Treatment of Acute Infections of Extremities, Minnesota Med. 21: 225, 1938.
12. Novak, M., and Hall, H.: Method for Determining the Efficiency of Preoperative Skin Sterilization, Surgery 5: 560, 1939.
13. Miles, G., and Farley, R. T.: Comparison of Effects of Sterile Antiseptic Jell With and Without Larval Ingredient on Wounds, Am. J. Surg. 45: 76, 1939.
14. Gruskin, B.: Chlorophyll—Therapeutic Place in Acute and Suppurative Disease, Am. J. Surg. 49: 49, 1940.
15. Dick, G. F.: Barberry—Ancient Remedy, New Germicide, Arch. Surg. 41: 287, 1940.
16. Tompkins, C. A., Crook, G. W., and Haynes, E.: Pectin in Treatment of Various Types of Wounds, Surg., Gynec. & Obst. 72: 222, 1941.
17. Bowers, W. F., and Kennedy, J. C.: Improved Management in Gangrene of the Foot, Am. J. Surg. 50: 573, 1940.
18. Keating, P. M., and Davis, F. M.: Prophylactic Treatment of Wounds in War, The Military Surgeon 86: 235, 1940.
19. Spink, W. W., and Paine, J. R.: Local Use of Sulfathiazole in Treatment of Staphylococcal Infections, Minnesota Med. 23: 615, 1940.
20. Jensen, N. K., Johnsrud, L. W., and Nelson, M. C.: Local Implantation of Sulfanilamide in Compound Fractures, Surgery 6: 1, 1939.
21. Varco, R. L.: University of Minnesota, Unpublished data.

WAR WOUNDS AND ANAEROBES

Lieut. Col. Roland I. Pritikin, M.C., 33rd Division, N.G.U.S.

(The writer states that the views expressed in this article are his own and that neither the Surgeon General nor the Medical Department of the Army is in anyway responsible)

HISTORY

Before we take up the study of anaerobes involved in war wounds let us view the history of the entire anaerobic realm.

In 1776 Spallanzani discovered that certain bacteria could live in an anaerobic environment. In 1861 Pasteur recognized the fact that certain organisms were able to exist and multiply in the absence of free oxygen.

The first of the pathogenic anaerobes to be discovered is the Leptothrix buccalis. Robin isolated and described it as early as 1847. In 1879 it was isolated by Trevesan. Frankel (1882), Michelson (1889), and Epstein (1900) observed leptothrix in connection with inflammations of the mouth and pharynx. Vignal and Arustamoff successfully isolated this organism.

Obermeier, engaged in the study of relapsing fever, isolated an organism which he called the spirillum of relapsing fever. This occurred in 1873. Little further work was done on this spirillum until thirty years later. From 1904 to 1908 various forms were discovered, among them the Spirocheta duttoni by Dutton and Todd in 1905; the Spirocheta dochii by Novy; the Spirocheta

Reprinted from The Military Surgeon 90: 164 (February), 1942.

carteri by Mackie; the Spirocheta vincenti by Blanchard. There are several types of relapsing fever and it is not yet known which types of spirochetes are responsible for the various types of the disease.

Schaudinn (1905) in his researches on Spirocheta pallidum very often found spirillum of very similar morphology. This spirillum is the Spirillum refringens, found in the mouth, tonsils, in ulcerating lesions, in smegma, and in venereal warts. Noguchi (1913), in cultivating various bacteria taken from lesions of the external genitals, discovered a new species, which he called Spironema phagedenae. Another similar anaerobic spirochete had been discovered in 1912 by King and Baeslake; they called it the Spironema hyos.

Framboesia tropica, a disease occurring in tropical and subtropical countries, was studied by Castellani (1905). He isolated from a large number of cases a spirochete which was named Spirocheta pertenue. It occurs in the cutaneous papules and ulcerations accompanying the disease. Von dem Borne repeated Castellani's investigations two years later and confirmed the facts stated by Castellani.

In the same year Schaudinn and Hoffmann carried on a series of investigations with syphilitic patients, examined primary syphilitic indurations and secondary enlarged lymph nodes, and discovered a spirochete which had not been described and which was found in infected syphilitic patients in a great proportion, and which was not found in uninfected patients.

Next to the Spirocheta pallidum comes the Clostridium tetani in interest from the standpoint of pathogenicity. Lockjaw had been known for many centuries, but it was not before 1883 that the causative agent of the disease was discovered. Carlo and Rattone in 1883 discovered it in the pus from a cutaneous lesion of a tetanus patient. They called it the bacillus of lockjaw. Nicolaier (1884) and Kitasato (1889), each carrying on investigations on tetanus, isolated and described a bacillus which is believed to be identical to the one described by Carlo and Rattone.

Van Ermergen (1896) investigated a portion of a pickled ham, the ingestion of which had caused disease of 34 persons, ten of them very seriously. He isolated an organism which he called the Bacillus botulinus. His work was confirmed by Romer (1900). In 1915 Nevin discovered another type of Bacillus botulinus, which he called Bacillus botulinus B.

Babes, Vincent, and Plaut are the chief workers on the disease known as Vincent's angina. In a throat smear of a patient suffering from Vincent's angina, Babes in 1889 isolated the Bacillus fusiformis.

A bacterium found in the stools of nursing children is the Bacillus bifidus, discovered by Tissier in 1900.

Less important are the following: The lymph node anaerobic bacillus, closely related to the bacteria group, also called the Corynebacterium, discovered by Torrey in 1916. The Bacillus egens, discovered by Stoddard in 1919. The multifermentans tenalbus, or Clostridium multifermentans, also discovered by Stoddard in 1919. The anaerobic pseudotetanus bacillus, or Clostridium tetanomorphum, discovered by MacIntosh and Fildes in 1917. Actinomyces necrophorus, discovered by Loffler in 1884, and the Treponema mucosum of Noguchi (1913).

A very extensive study was made of the anaerobic organisms associated with traumatic injuries during World War I. Most of the bacilli had been discovered long before the outbreak of the war, but it was during the war that we learned so much about them. The first of these organisms to be discovered was the Vibrion septique of Pasteur and Joubert (1877). B. welchii found in gas gangrene, usually in civilian cases, was first discovered by Welch and Nuttall (1892). This bacillus has also been called the gas bacillus, Bacillus perfringens, and Clostridium welchii. Another bacillus of this group is B. oedematiens, which infected a large number of wounds, according to the discoverers, Weinberg and Sequin (1915). In the same year Weinberg and Sequin isolated B. fallax, a slightly pathogenic anaerobe. The following year they discovered the B. histolyticus, an intensely proteolytic anaerobe found associated with the anaerobes in war wounds. In 1889, twelve years after Pasteur had discovered Vibrion septique, Cornevin, Arloing, and Thomas found a bacillus which they called the Bacillus chauvoei, the bacillus of symptomatic anthrax or blackleg.

Another organism that Weinberg and Sequin claim to have occurred frequently in war wounds is the B. sporogenes, discovered by Metchnikoff in 1908. In putrid wounds is occasionally found B. putrificus, discovered by Bienstock (1884). The B. sporogenes cadaveris of Klein is perhaps the same as B. putrificus. Tissier and Martally have found this organism present in putrid meat. The Bacillus oedematiens of Weinberg and Sequin is similar to, but not identical with, Bacillus oedematiens II, or bacillus of Novy, discovered by Novy in 1894.

TECHNIQUE OF ISOLATION

Anaerobic bacilli are comparatively difficult to isolate. It was not until World War I that efficient methods for the isolation of anaerobes were developed. The early workers usually were working with several organisms when they thought that they had a pure culture. MacIntosh and Fildes developed a good method for their isolation by using a technique which involved repeated plating of the anaerobic bacilli. Veillon's method was used by some workers. This consisted of inoculating agar shake tubes with varying dilutions of material. The tube is then filed through at the level of the colony, and the colony is then fished. The best method is Barber's method. (Developed by Barber in the laboratories of the Rockefeller Institute.)

In Barber's method a pipette is used to pipette out the colony. Barber claims that his method is not as time-consuming as the older methods, and permits the observer to make further observations on anaerobiosis, variability, motility, rate of growth, and behavior in a medium of small sowing exactly known than the older methods allowed.

An isolation chamber is used, 7 cm. long, 3.25 cm. broad, and 2 cm. high, which gives sufficient room and allows the use of a large cover glass, 60 by 35 mm. The cover glass must be heated very clean, because it may be used for growing colonies on it. It is carefully cleaned, smeared with vaseline, washed with hot water and alcohol, and wiped with a clean cloth. This leaves a very thin film on the glass. If sterilization is necessary, it should be done over a flame of low temperature so as not to melt the vaseline. The point of

the pipette should be made very fine, just large enough to permit the entrance of organisms. It should be about 5 micra in diameter.

With the aid of a microscope a small droplet is pipetted from a cover slip containing a thin film of the solution with the organism to be isolated. The droplets pipetted off should then be examined under oil immersion. This will insure the purity of the culture insofar as organisms visible to the oil-immersion lens are concerned. Invisible organisms cannot be guarded against.

The isolated organisms are then transferred to test tubes, or grown on cover glasses and then transferred. A fresh pipette is used for each organism, and the organisms are separately transferred to test tubes. Each new pipette has the tip sterilized by supplying same with a small amount of sterile broth before use. This aids also in washing the bacilli well into the tubes in the case where bacilli adhere to vaseline on the outside of the pipette. In washing the bacilli out, the technician must be careful not to blow any air into the culture. The pipette method is used upon seeding material isolated by the usual means. Air was removed by pumping in oxygen or by heating, the culture being covered by a layer of vaseline ⅕ to 2 cm. thick which prevents access of air. For growing spores in hanging drops, Barber used the following method: A shallow moist glass chamber, 45 mm. long, 25 mm. broad, and 2 mm. deep, is made. The isolated spores are arranged on a cover glass, 1.5 or 2 mm. apart, and sufficient media added. The undersurface of the cover glass, with the exception of the central portion containing the isolated spores, is then covered with a soft glucose agar culture of B. pyocyaneus, about 4 or 5 hours old. The area in the center may be surrounded by a thin paraffin wall to prevent spreading of the pyocyaneus. The bottom of the moist chamber is also supplied with a similar layer of pyocyaneus culture. Pyrogallic acid and KOH were used by Barber in connection with B. pyocyaneus, but they did not offer any advantage. Barber made sowings of the following bacilli: B. sporogenes, B. welchii, B. tetani, B. edematis, B. botulinus, B. of Ghon Sachs, B. aerofitidus, B. putrificus, B. bellonensis, B. tertius, B. fallax, B. edematiens, B. bifermentans, B. histolyticus, Vibrion septique.

Out of 400 one-cell sowings of bacilli, he succeeded in producing growth in 62, or 15.5 per cent.

He made 211 one-cell sowings, using all of the above, with the exception of welchii, edematis, aerofetidus and fallax and producing growth in 93, thus getting as a result 44.1 per cent. For the bacilli he used as media glucose broth, serum glucose broth, semisolid glucose agar, semisolid serum agar, firm glucose agar, firm Veillon agar, liver peptone agar, minced brain, liver peptone water under vaseline, liver peptone water in vacuum, milk and plain broth. For the spores he used in addition, egg cube and meat, but did not use liver peptone agar or minced brain. The bacilli, as a whole, grew best on the various glucose media, the spores on the semisolid glucose agar. Barber also found that spores remained viable after exposure for an hour or more, whereas bacilli were much more rapidly affected, depending on species. Barber found that B. sporogenes grows very rapidly, B. bellonensis very slowly.

In summarizing, Barber made the following statements: "(1) The pipette method has proved a feasible method of obtaining pure cultures of one-cell

anaerobes. (2) Both bacilli and spores may be used as seeding material, but spores give a much higher percentage of positives. Boiling alone affords a sufficient degree of anaerobiosis to the medium for initiating one-cell growth; and semisolid agar is the most convenient form of medium. Exposure to air during isolation apparently has no effect on the viability of spores of anaerobes, but young bacilli of some species suffer from a comparatively short exposure to free oxygen."

CULTURAL CHARACTERISTICS

The cultural characteristics of the anaerobic bacilli causing gas gangrene will be discussed. We may divide these up into two groups, saccharolytic and proteolytic, as follows: saccharolytic, B. welchii, Vibrion septique, B. oedematiens, B. fallax; proteolytic, B. sporogenes, B. histolyticus, B. putrificus.

B. welchii is a gram-positive, nonmotile organism. In older cultures it may become gram-negative. Its length is 4 to 8 micra, breadth 1 to 1.5 micra, straight and usually square. It may be coccus-shaped or rod-shaped, and has a capsule. It has no flagella, no spores, and is not very strictly anaerobic. The surface colonies on nutrient agar, serum agar, or glucose agar are circular in contour. Old colonies are 1 to 2 mm. in diameter. Young colonies, that is, colonies about 12 hours old are translucent. It ferments the common sugars, starch, and may ferment glycerin and inulin. It does not ferment mannite, dulcite, salicin. It grows rapidly on meat, with a formation of acid and gas. In milk we get what is known as stormy fermentation. Acid and gas are formed with remarkable rapidity in great quantities. No spores. In coagulated serum there is no change, no liquefaction, and spores are formed. In alkaline egg broth an opacity results. Gelatin is liquefied in 48 hours in an inoculated tube at 37 degrees Centigrade.

Vibrion septique is gram-positive, composed of slender rolls, strictly anaerobic, has spore formation, is motile, has no capsule. On meat we get a rancidity and gas. On milk, where it is usually found, it causes acidity, produces a little gas, and causes clotting. There is no liquefaction on coagulated serum. Alkaline egg broth growth results in opacity, but there is no clotting. It liquefies gelation. When grown on broth, a turbidity forms which settles to the bottom, leaving a clear supernatant fluid. It will ferment glucose, levulose, galactose, maltose, lactose, and salicin. It will not ferment glycerin, saccharose, inulin, mannite, and dulcite.

It may be differentiated from the B. chauvoei of Arloing, Cornevin, and Thomas by the fact that Vibrion septique will ferment salicin; B. chauvoei will not.

B. oedematiens is motile under strictly anaerobic conditions. It is a stout rod, 0.8 to 1 micron in length, which is somewhat wider than B. welchii. The rod is lightly curved. Autolysis sets in very early in B. oedematiens cultures. Strict anaerobic conditions for good growth are required. In meat we get gas; in milk, acid, and at the end of 4 or 5 days, a clot. Coagulated serum, no change. Broth, a flocculation and a semi-opaque cloud. Gelatin is liquefied. The production of acids is feeble in fermentations. It will ferment glucose, levulose, and maltose. It will not ferment glycerin, galactose, saccharose, lactose, mannite, dulcite, inulin, and salicin.

B. fallax is a motile, slender rod, 3 to 6 micra in length, has rounded ends, and is slightly curved. Gram-negative elements are frequent. When colonies are grown, those on the surface appear as round, crenated, slightly granular. The deep colonies are lenticular, irregular, or bean-shaped. It will not digest meat, but will form acid. In three to seven days a milk culture clots. With coagulated serum there is no liquefaction. The same is true of gelatin. All strains of B. fallax will ferment glucose, levulose, and maltose, but only a few strains will ferment galactose, saccharose, starch, inulin, and salicin.

B. sporogenes is ancillary to the condition of gas gangrene. It is actively motile, slender, forms spores, gram-negative in old cultures, gram-positive in young cultures, and is not absolutely anaerobic. The colonies have a wooly, tangled filament structure on the periphery, and have a hard center. The deep colonies are wooly. On meat there is a vigorous growth, with gas formation, an alkaline reaction, and a putrid odor. In milk a turbidity is formed which settles out, leaving a clear supernatant fluid, the reaction becoming alkaline. Coagulated serum is liquefied. In alkaline egg broth a flocculent precipitate falls to the bottom and is digested. Gelatin is liquefied. It will ferment only glucose, levulose, and maltose. Some of the other organisms ancillary to a condition of gas gangrene are: B. cochlearius, B. parasporogenes (McIntosh, 1917), B. tertius (Henry, 1917, Rodell as B. III of von Hibler as B. IX), B. tetanomorphus, B. aerofetidus, B. bifermentans, B. putrificus, B. butyricus, B. multifermentans tenalbus.

B. histolyticus is motile, rod-shaped, frequently arranged in pairs, is 3 to 5 micra in length and 0.5 to 0.84 broad. Cultural reactions are characteristic for the group. The surface colonies are delicate, flat, crenated, and have irregular edges. In agar the deep colonies are arborescent or corallike, with fine wooly ends to the branches. A white deposit of tyrosine follows digestion upon inoculation in meat. Coagulated serum is liquefied and gelatin is liquefied. Glucose, levulose, maltose-fermented.

B. putrificus is strongly proteolytic, gram-positive rod, oval spores, strictly terminal, giving drumstick appearance. Gelatin and serum are liquefied; milk is digested. It is found in gas gangrene and contributes to the putrid odor, but is in itself not pathogenic.

POWER OF RESISTANCE

In the classification of anaerobes we inquire as to their resistance to an oxygen environment. In viewing the bacterial realm, we notice that we have various groups as regards aerobism. To one of these groups atmospheric oxygen is a toxin. This group is the obligative anaerobe group. Barber's experiments showed that the presence of free oxygen stopped growth as follows: B. welchii, 23 minutes; B. tetani, 60 minutes; B. oedmatis, 10 minutes; B. botulinus, 6 minutes; B. sporogenes, 0 minutes; B. of Ghon Sachs, 6 minutes; B. aerofitidus, 10 minutes; B. putrificus, 1 minute; B. bellonensis, 6 minutes; B. tertius, 10 minutes; B. fallax, 6 minutes; B. oedematiens, 17 minutes; B. bifermentans, 6 minutes; B. histolyticus, 10 minutes.

No culture has ever been grown absolutely oxygen free; a little oxygen is always present. Anaerobes live symbiotically with aerobes to a great extent, and perhaps acquire oxygen in that fashion.

anaerobes. (2) Both bacilli and spores may be used as seeding material, but spores give a much higher percentage of positives. Boiling alone affords a sufficient degree of anaerobiosis to the medium for initiating one-cell growth; and semisolid agar is the most convenient form of medium. Exposure to air during isolation apparently has no effect on the viability of spores of anaerobes, but young bacilli of some species suffer from a comparatively short exposure to free oxygen."

CULTURAL CHARACTERISTICS

The cultural characteristics of the anaerobic bacilli causing gas gangrene will be discussed. We may divide these up into two groups, saccharolytic and proteolytic, as follows: saccharolytic, B. welchii, Vibrion septique, B. oedematiens, B. fallax; proteolytic, B. sporogenes, B. histolyticus, B. putrificus.

B. welchii is a gram-positive, nonmotile organism. In older cultures it may become gram-negative. Its length is 4 to 8 micra, breadth 1 to 1.5 micra, straight and usually square. It may be coccus-shaped or rod-shaped, and has a capsule. It has no flagella, no spores, and is not very strictly anaerobic. The surface colonies on nutrient agar, serum agar, or glucose agar are circular in contour. Old colonies are 1 to 2 mm. in diameter. Young colonies, that is, colonies about 12 hours old are translucent. It ferments the common sugars, starch, and may ferment glycerin and inulin. It does not ferment mannite, dulcite, salicin. It grows rapidly on meat, with a formation of acid and gas. In milk we get what is known as stormy fermentation. Acid and gas are formed with remarkable rapidity in great quantities. No spores. In coagulated serum there is no change, no liquefaction, and spores are formed. In alkaline egg broth an opacity results. Gelatin is liquefied in 48 hours in an inoculated tube at 37 degrees Centigrade.

Vibrion septique is gram-positive, composed of slender rolls, strictly anaerobic, has spore formation, is motile, has no capsule. On meat we get a rancidity and gas. On milk, where it is usually found, it causes acidity, produces a little gas, and causes clotting. There is no liquefaction on coagulated serum. Alkaline egg broth growth results in opacity, but there is no clotting. It liquefies gelation. When grown on broth, a turbidity forms which settles to the bottom, leaving a clear supernatant fluid. It will ferment glucose, levulose, galactose, maltose, lactose, and salicin. It will not ferment glycerin, saccharose, inulin, mannite, and dulcite.

It may be differentiated from the B. chauvoei of Arloing, Cornevin, and Thomas by the fact that Vibrion septique will ferment salicin; B. chauvoei will not.

B. oedematiens is motile under strictly anaerobic conditions. It is a stout rod, 0.8 to 1 micron in length, which is somewhat wider than B. welchii. The rod is lightly curved. Autolysis sets in very early in B. oedematiens cultures. Strict anaerobic conditions for good growth are required. In meat we get gas; in milk, acid, and at the end of 4 or 5 days, a clot. Coagulated serum, no change. Broth, a flocculation and a semi-opaque cloud. Gelatin is liquefied. The production of acids is feeble in fermentations. It will ferment glucose, levulose, and maltose. It will not ferment glycerin, galactose, saccharose, lactose, mannite, dulcite, inulin, and salicin.

B. fallax is a motile, slender rod, 3 to 6 micra in length, has rounded ends, and is slightly curved. Gram-negative elements are frequent. When colonies are grown, those on the surface appear as round, crenated, slightly granular. The deep colonies are lenticular, irregular, or bean-shaped. It will not digest meat, but will form acid. In three to seven days a milk culture clots. With coagulated serum there is no liquefaction. The same is true of gelatin. All strains of B. fallax will ferment glucose, levulose, and maltose, but only a few strains will ferment galactose, saccharose, starch, inulin, and salicin.

B. sporogenes is ancillary to the condition of gas gangrene. It is actively motile, slender, forms spores, gram-negative in old cultures, gram-positive in young cultures, and is not absolutely anaerobic. The colonies have a wooly, tangled filament structure on the periphery, and have a hard center. The deep colonies are wooly. On meat there is a vigorous growth, with gas formation, an alkaline reaction, and a putrid odor. In milk a turbidity is formed which settles out, leaving a clear supernatant fluid, the reaction becoming alkaline. Coagulated serum is liquefied. In alkaline egg broth a flocculent precipitate falls to the bottom and is digested. Gelatin is liquefied. It will ferment only glucose, levulose, and maltose. Some of the other organisms ancillary to a condition of gas gangrene are: B. cochlearius, B. parasporogenes (McIntosh, 1917), B. tertius (Henry, 1917, Rodell as B. III of von Hibler as B. IX), B. tetanomorphus, B. aerofetidus, B. bifermentans, B. putrificus, B. butyricus, B. multifermentans tenalbus.

B. histolyticus is motile, rod-shaped, frequently arranged in pairs, is 3 to 5 micra in length and 0.5 to 0.84 broad. Cultural reactions are characteristic for the group. The surface colonies are delicate, flat, crenated, and have irregular edges. In agar the deep colonies are arborescent or corallike, with fine wooly ends to the branches. A white deposit of tyrosine follows digestion upon inoculation in meat. Coagulated serum is liquefied and gelatin is liquefied. Glucose, levulose, maltose-fermented.

B. putrificus is strongly proteolytic, gram-positive rod, oval spores, strictly terminal, giving drumstick appearance. Gelatin and serum are liquefied; milk is digested. It is found in gas gangrene and contributes to the putrid odor, but is in itself not pathogenic.

POWER OF RESISTANCE

In the classification of anaerobes we inquire as to their resistance to an oxygen environment. In viewing the bacterial realm, we notice that we have various groups as regards aerobism. To one of these groups atmospheric oxygen is a toxin. This group is the obligative anaerobe group. Barber's experiments showed that the presence of free oxygen stopped growth as follows: B. welchii, 23 minutes; B. tetani, 60 minutes; B. oedmatis, 10 minutes; B. botulinus, 6 minutes; B. sporogenes, 0 minutes; B. of Ghon Sachs, 6 minutes; B. aerofitidus, 10 minutes; B. putrificus, 1 minute; B. bellonensis, 6 minutes; B. tertius, 10 minutes; B. fallax, 6 minutes; B. oedematiens, 17 minutes; B. bifermentans, 6 minutes; B. histolyticus, 10 minutes.

No culture has ever been grown absolutely oxygen free; a little oxygen is always present. Anaerobes live symbiotically with aerobes to a great extent, and perhaps acquire oxygen in that fashion.

During World War I it was forcibly called to the attention of the clinicians that the resistance of the bacteria to certain antiseptics did not have the same effect on the bacteria in the wound as it did in the laboratory.

Antiseptics of rapid action, which anaerobic bacteria are unable to resist, are Dakin's solution, eusol (hypochlorite solutions), and chloramine T. These antiseptics prevent further growth even in optimum media. With eusol the following figures will show result of action:

TIME EUSOL REMAINED IN WOUND	NUMBER OF COLONIES DEVELOPING	NUMBER OF BACTERIA PER C.C. OF FLUID IN WOUND, CALCULATED
5 minutes	2	200,000
20 minutes	50	5,000,000
30 minutes	40	4,000,000
60 minutes	25	2,500,000

TIME CHLORAMINE T IN WOUND	NUMBER OF COLONIES DEVELOPING ON PLATES
10 minutes	No growth after 24 hours Innumerable colonies after 4 days
20 minutes	Innumerable colonies after 4 days
30 minutes	Innumerable colonies after 48 hours
60 minutes	Innumerable colonies after 48 hours

The antiseptics used above are very destructive to anaerobes in the laboratory but their potency is dissipated in some way in the wound perhaps by contact with the walls of the wound, its discharges, or both of these factors. By experiments carried on in vitro it was discovered that the bactericidal efficacy of antiseptics is lessened in the presence of serum, and that leucocytes have a quenching effect upon antiseptic agents. The values for the concentrations of various drugs to inhibit completely the growth of B. sporogenes are as follows: Iodine 1:500; chloramine T 1:50; carbolic acid 1:200; mercuric chloride 1:2,000; crystal violet in 6,400. For B. welchii the values are as follows: Iodine 1:500; eusol 1:4; chloramine T 1:60; carbolic acid 1:200; mercuric chloride 1:2,000; malachite green 1:200; flavine 1:16,000; crystal violet 1:1,000. Dyestuffs in general exert an inhibitory effect on the bacteria. The bactericidal dyes lose a great deal of their potency by combining with the fabrics used for bandaging.

Anaerobes, and especially their spores, are comparatively resistant to desiccation. Light is a strong bactericidal agent, but in the case of the anaerobes, a completely anaerobic environment will interfere with the bactericidal action of light. Hydrogen peroxide is a strong bactericidal agent.

A hypertonic salt solution is an indirect bactericidal agent; it produces an accumulation of leucocytes. Electric light, x-rays, and radium light have an inhibitory effect on anaerobic growth. Electrical currents applied to a solution containing anaerobes will act as a bactericidal agent. Heat will kill anaerobes.

CHANNEL OF INFECTION—MOST COMMON SOURCE

The point of infection is the wound. Of the various types some are more liable to anaerobic infection than others. The more irregular and lacerated wounds become more highly infected. In the case of a wound caused by a shell particle acute infection follows for two reasons: The shell particle makes a rougher wound than the high-powered bullet, and the shell bursting on percussion with the ground carries with it as it enters the wound portions of soil,

which in highly fertilized districts may be heavily charged with anaerobes. Infection may take on various phases. We may get a localized anaerobic infection in the wound, slowly spreading anaerobic infection in the wounds; gas gangrene of the "group" type when a single muscle or group of muscles is attacked; gas gangrene of the massive type when a whole segment of a limb is involved; or the fulminating type.

Infection is aided by the following factors: Retention of extravasated blood; interference with the local circulation; the presence of masses of devitalizing tissues; extensive fractures and comminution of long bones; retention of wound secretions by dressing, pastes, or packings. The wound, if it has the shape of a cavity with a small surface opening, is ideal for anaerobic growth. Delay in the mechanical cleansing of a wound leads to acute infection, as does also the retention of foreign bodies.

PATHOGENICITY

In a wound a myriad of factors enter which determine whether an anaerobic infection will be apparent within an hour, or whether several days will elapse before distinctive features appear. An infection usually takes the following course: The wound has a brownish discharge, composed of broken down blood clots; it is foul smelling and gives off gas. The odor depends on the infecting organism and the stage of the wound. At the very beginning a characteristic smell is absent, but later it becomes foul and acrid. A serous infiltration occurs throughout the intermuscular septa and connective tissue. The accumulation of the fluid causes a swelling which takes on the appearance of a purplish ring around the wound. The muscle is at first not infected; when infection sets in, we have the condition of gas gangrene. The muscle tissue is invaded by the organisms and is gradually digested. If the blood supply is left intact, we are able to see the actual course of infection. The muscle becomes black, friable, and diffluent. Then we get several color zones, first red, then yellow. Contractility is lost and the muscle undergoes changes in coloring, beginning with red and changing to greenish yellow and then to black.

In another type the blood supply is cut off. The muscle changes color from purplish red to a greenish black diffluent mass. Gas forms first as bubbles between the muscle fibers, afterwards entering the areolar tissues. The gas has a deleterious effect, since it produces pressure in the fascial sheath and constricts the blood vessels. Interference with the blood supply is very favorable to infection, because neither the first nor second line of defense is given free play.

Toxin Production.—B. welchii produces a true exotoxin. An antitoxin was first developed by Klose (1916), which was not, however, as effective as Bergston's. Since B. welchii is the chief organism involved in gas gangrene, an antitoxin injection will remove the organism causing the most trouble, and will leave the minor gas gangrene organisms alone, among them the Vibrion septique and B. oedematiens, which come next on the list from the point of view of infection. B. welchii was isolated by Weinberg and Sequin in 72 to 80 per cent of the cases of gas gangrene studied by them during the war.* Pathogenicity and hemolytic power vary greatly with the different strains of B.

*World War I.

welchii. In fatal cases death usually results from a B. welchii septicemia. Spores are never formed in the animal body.

Vibrion septique has a very powerful toxin. It is a soluble toxin. A specific antitoxin has been developed.

B. oedematiens is, in general, pathogenic, although two nonvirulent strains have been isolated by Weinberg and Sequin. The lesion caused by B. oedematiens is characterized by a whitish, gelatinous exudate and absence of gas. B. oedematiens forms a soluble toxin, but does not cause the acute infection that Vibrion septique does.

B. fallax is very slightly pathogenic.

B. sporogenes was found in 27 per cent of the cases examined by Weinberg and Sequin; it is usually responsible for the foul odor in wounds. This organism is, however, not very pathogenic and produces a weak toxin.

B. histolyticus is intensely hemolytic and produces large lesions, but is nontoxic, and does not produce acute infections.

B. putrificus is actively proteolytic, produces foul odor and putridity in wounds, and is nonpathogenic. The condition of the wound under treatment may become better or worse. If better it heals; the pus is drained and granulation sets in. If the wound becomes worse, the gas gangrene condition rapidly encompasses more area, and we get a condition which demands amputation.

The infection, although primarily caused by anaerobic bacilli, is usually found to contain aerobes, usually streptococcus and staphylococcus. The anaerobes grow better under an environment containing aerobes. Thus to remove the aerobes, which are aiding in causing a media favorable to anaerobes, we inject some staphylococcus vaccine, or a vaccine made from M. tetragenus and streptococcus. The vaccine usually improves the wound. Vaccine gave good results in cases where a condition ensued in which there was a pyrexia, a cessation of the healing process, and an unhealthy appearance in the wounds. In the treatment we must be careful of our use of antiseptic: a very weak solution of chloramine T, for example, will have a stimulating effect upon the organisms.

DISSEMINATION WITHIN THE BODY OF THE HOST

In the case of gas gangrene, the condition is such that dissemination of the infective material to even a comparatively small extent is fatal. The treatment of a wound is determined by the danger of dissemination to a very great extent. If we have the wound in a limb, the limb must be amputated if the infection cannot be controlled. We give our antiseptics with the view of preventing spread of infection. Thus, when a patient is brought into the hospital, the wound is immediately washed with hypochlorite solution, and we apply eusol, Dakin's solution, or chloramine T. Hypochlorite solution is the most effective means for putting an initial stop to dissemination.

Slough-covered wounds are given frequent washings with a 0.5 per cent salt solution, which causes a liberation of a tryptic ferment upon the disintegration of the leucocytes in pus. An exudation of lymph also ensues. I will give a brief summary of a few cases showing dissemination of the disease. (Great Britain Medical Research Council Report 57: 122-150, 1920.)

I. Bullet entered right thigh, gluteal muscles affected by gas gangrene, no healing, general condition worse—amputation.

II. Gangrene in muscle of leg. Dissemination stopped by hypochlorite applications. Connective tissue repair.

III. Wounded. Was lying on the field for three days before he was picked up. He was very weak from loss of blood, and an infection spread in his leg. He was taken to the base hospital. Wound was treated with Dakin's solution, and every attempt was made to stop spread of infection. Infection continued spreading so that leg was amputated above the knee.

IV. He was wounded in the forearm. Incisions were made for gas gangrene. Infection was counteracted. Granulation tissue repair. Muscle was not as efficient as formerly after wound was healed.

V. Wound in right scapula. Incisions made on surface with extra precautions. Easily repaired. This type of wound offers comparatively little trouble.

VI. Wounded in leg. Muscle became gangrenous and was excised. Rapid recovery followed, although leg was not as efficient as formerly.

VII. Infection in gluteal muscles. Incisions were made into muscle, but did not aid in stopping spread of infection. Muscles excised. Fever followed, and large pieces of necrosed bone were removed from the neighborhood of the sacroiliac joint, which greatly improved the drainage of the deeper portion of the wound. The wound progressed wonderfully quickly, granulations springing up from the bottom and filling the cavity, the epithelium also spreading in from the edges. When later the concentration of material became great and contraction followed, but was not rapid enough, and epithelium stopped spreading, some 45 Steele's grafts taken from the patient's thigh were applied to the granulating surface, no special arrangement being adopted. A second skin grafting operation two months later when the scar tissue was very pronounced, and the granulations in consequence very bloodless, was an almost complete failure. However, under massage to loosen the scar tissue, and to promote circulation, healing progressed slowly and patient was discharged.

VIII. Bullet going through and through, leaves clean wound, very easily healed.

IX. Femur fractured. Drainage incisions made to curb infection. Infection stopped. Operation, binding bone with metallic thread. Operation for removal of metallic thread. Wound heals.

X. Infection attacks to the extent of septicemia, in very weak patient. Death.

XI. Pus from wounds in head drained. This condition is very dangerous due to proximity to the brain.

XII. Repeated amputations. Pleurisy set in. Death due to either septic thrombosis of the vena cava or septic thrombosis of the inferior or common iliac vein.

XIII. Wound healing. Severe secondary hemorrhage, wound opened, but bleeding point could not be found due to infiltration of blood and inflammatory exudation. Amputation found necessary and performed.

In general, we may say this about dissemination. An infection is started and we have an inflammation. Then there is a leucocytic infiltration. Necrosis sets in, muscle and bone tissue are destroyed, there is a lymphocytic infiltration, and a putrefied mass is formed in the wound. The wound has a tendency to close on the surface, leaving a putrid proliferative mass. A flesh wound on the abdomen, if severe enough, may gain entrance into the abdominal cavity. A case of gangrene in the abdominal region is rare, due to the fact that abdominal flesh wounds are easily treated. Dissemination of infection of a wound of this kind into the abdominal cavity is usually fatal.

Thrombosis, embolism, septicemia often result in cases of gas gangrene, the latter being fatal, the former two leading to a critical condition.

Various complications may arise. In gas gangrene septicemia a high temperature is reached (102 to 103 degrees Fahrenheit) and death follows. Very rarely we may get a gas gangrene pyemia. There is an invasion into the blood stream, secondary deposits of gas gangrene bacilli in various parts of the body when the tissues have suffered some slight damage, as from the introduction of a hypodermic injection, or an infusion of saline, or they may develop in tissues subjected to prolonged pressure as in the buttock, when the patient lies in bed tilted on one hip. The prognosis is extremely bad.

CLINICAL SIGNIFICANCE

A. *Diagnosis of the Disease From a Clinician's Standpoint*

1. *The physical standpoint:* The physical signs differ according to the type of the disease present. A localized anaerobic infection is distinguished by a foul-smelling discharge, mixed with bubbles of gas. If the disease is more extensive, swelling is noted, and upon percussion of the swollen area a tympanitic sound is heard.

By noting the type of wound, we are able to tell whether an anaerobic infection will soon be in evidence. Wounds with imperfect drainage, extensive devitalization, and death of tissue with extravasation of blood, will result in gas gangrene cases. Imperfect drainage is typical in cases where a projectile made a small point of entrance, entered deeply, and was retained. The general condition of the wounded man is also important. Infection develops much more readily in fatigued troops than in fresh troops. An irregular projectile forms a more irregular wound, and may carry bits of clothing, and thus increase the chances of infection. An area with a diminished blood supply precludes active intervention by the protective mechanisms of the body. When a wound becomes infected, it is an anaerobic infection up to the time the muscle is attacked, then it becomes gas gangrene. The muscle becomes black, friable, and diffluent, and the line of invasion is seen. In others we see red and yellow zones or bands. In some our chief symptom is a loss of contractility. The diagnostic features of gas gangrene are pain, crepitation, resonance on percussion, and what is more important, severe constitutional symptoms.

The disease may be one of several types. In the first type we have a localized anaerobic infection. In the second type we have slow spreading. In the third, swelling, and a tympanitic condition and crepitation may be detected. These symptoms become more marked as the disease continues, the skin develops large irregular fibrillae, with blood-stained serous fluid. There is a mottling of purple patches, becoming greenish yellow. The muscle becomes dry, brown, pultaceous, and finally black with a slimy surface. This third type is called the group type. The fourth is the massive type. Here the blood supply is cut off, constitutional symptoms, such as vomiting, rise in temperature, and a rapid pulse are noted. There is a rapid appearance of signs of decomposition. The fifth type is the fulminating type. Severe pain, extensive swelling of the affected part, rapid spread of the disease, and severe constitutional features are the symptoms.

2. *Symptoms:* In the early stages pain is very marked; the unusual amount of pain depends upon the increasing pressure within the wound. When the

infection becomes established, pain increases. There is a feeling of numbness in the superficial parts of the limb. The patient looks distressed and ill. Lips acquire a cyanotic color, the pulse rate is rapid, and the temperature rises 3 to 4 degrees Fahrenheit. There is continuous vomiting. If the disease becomes more rapid, becomes running and uncontrollable, there is more frequent vomiting, the extremities become cold and blue, the temperature falls. The mind, however, remains acute even to the end; in the terminal stages some degree of general icterus may be present. Death comes with dramatic suddenness; the specific cause is still unknown. Pulmonary embolism is never found in autopsy.

B. Laboratory Standpoint

The primary infection is largely made up of fecal material bacteria. B. welchii and associated anaerobes in symbiosis with aerobes, chiefly streptococcus and staphylococcus. After the healing process had begun and granulation tissue formed, no B. welchii were found upon laboratory examination. In recrudescence of bacterial infections after operations, the chief anaerobe to be found was B. sporogenes, always in company with streptococci and staphylococci. As soon as a case was brought into the hospital and physical diagnosis was made, samples of pus were collected and films and cultures were made. The specific organisms causing the infection are thus noted. Weinberg and Sequin give the following figures: B. welchii were found in 72-80 per cent of cases; Vibrion septique in 12 per cent of cases; B. oedematiens in 34 per cent of cases; B. sporogenes in 27 per cent of cases.

REFERENCES

Texts: Hiss-Zinsser, Jordan, Kendall.
Barber, M. A.: J. Exper. Med. 32: 295-311, 1920.
Great Britain National Health Insurance, Medical Research Council 39: 1-182, 1919. Committee—Bulloch, Douglas, Henry, McIntosh, O'Brien, Robertson, and Wolf.
Great Britain Medical Research Council Report 57: 1-122, 1920. Authors—Douglas, Fleming, Colebrook.

NEWER CONCEPTS IN THE TREATMENT OF BURNS*

WITH SUGGESTIONS FOR THE MANAGEMENT OF WARTIME THERMAL INJURIES

LIEUT. THEODORE A. FOX, M.C., U.S.N.R.

IN 1925, when the tannic acid treatment of burns was introduced a remarkable step forward was made in reducing the morbidity and mortality of thermal injuries.

It was soon discovered that tanning (with the later modification of applying silver nitrate to the area) relieved pain by protecting exposed nerve endings and stopped the escape of precious body fluids by forming a damming eschar over the raw area. Both were important factors in reducing shock. However, the method had two marked disadvantages.

In the first place, it was necessary to scrub the patient thoroughly before applying the tannic acid spray, for every effort had to be made to cleanse and "sterilize" the skin before applying, because tannic acid is not bactericidal. Infection and associated toxemia, once started beneath the tough protecting but masking eschar, would often be the cause of the patient's death. This dire complication is usually due to contamination of the raw surfaces before the tanning takes place and the eschar forms. The burned area is sterile for some time after the injury, since bacteria are killed by the same physical cause that kills cells and tissues. Toxemia is the second most important complication as a cause of death from burns; shock is first.

Secondly, when the epithelization occurred under the tough, inflexible eschar, the cells of epithelium that grow into the burned area from the unburned margins were not "normal" in that they, too, had been "tanned." The connective tissue beneath, instead of being elastic, forms tough, contracted bands under the deformed epithelium. This grave aftermath, usually of third-degree burns, frequently causes more concern than the original injury, especially if the face, neck, hands, or flexor surfaces of the extremities are involved. The patient recovers from the burn but is frequently left a deformed, crippled monster. Infection further increases the scarring, even though sulfon-chemotherapy has reduced the morbidity. The patient faces months of painful and expensive plastic surgery in order to return to a semblance of normality.

Not only is tannic acid (even with silver nitrate) nongermicidal, but it has the added disadvantage of being extremely unstable, necessitating fresh solutions whenever needed. Such an eschar is thick and likely to crack, and if the surface to be tanned is already infected, it will not "take."

*Reprinted from the U. S. Nav. Med. Bull. 40: 557 (July), 1942.

It was plainly seen after years of experience with this treatment that for the reasons discussed, the tannic acid method could be improved upon. What other methods could be used to give an antiseptic, analgesic eschar that would provide a dam for escape of tissue fluids, stimulate normal epithelization, and form a nondisfiguring scar?

Aniline dyes had been used in connection with burns for years; singly they did not seem to have the proper qualities to displace tanning.

In 1933 Aldrich[1, 2] happened upon the ideal combination of these dyes. He showed by culturing burns that they were sterile (due to thermal injury of the skin) up to eighteen hours after the injury occurred. If the patient showed no signs of toxemia after this, the wound remained relatively sterile. Up to the seventy-second hour a mixed type of infection could be cultured from the wound surface; and after that period, pure cultures of beta and gamma hemolytic streptococci could be demonstrated in a toxic patient. Aldrich felt that the infection was the cause of this early toxemia, rather than the previously suggested split proteins, and he was able to prove his thesis experimentally, not only by surface culture of the burn, but from blood cultures as well; he also showed that only infected cases became toxic. This led him to search for an eschar-forming dye that was noninjurious to the tissues, yet strongly germicidal. He found that gentian violet was effective against the gram-negative organisms. But it was not until he mixed crystal violet and neutral acriflavine (acriviolet) and brilliant green that he was able to kill the gram-positive organisms as well as the gram-negative, yet not injure tissues. Not only did these dyes in combination (1:1,000 solution) kill all germs; they formed a tough, purple-brown, supple eschar to seal the area, and provided excellent analgesia as well.

If a local area becomes infected in spite of the triple-dye treatment, it is not masked until late in the course of this complication, as it is in the tannic acid method. By daily observation a softened, moist area can readily be detected and by simply trimming it out and respraying the denuded area, one can quickly end this complication.

The three-dye mixture consists of 1 per cent aqueous solution of crystal violet (gentian violet also is used) and brilliant green, and a 0.1 per cent solution of neutral acriflavine. This mixture is quite stable and can be prepared and stored for a long time without losing its efficiency. The crystal violet is specific for gram-positive organisms and has a definite analgesic action in that it coagulates with plasma proteins to form the eschar protection for the exposed nerve endings. The neutral acriflavine and the brilliant green dyes have special germicidal action against gram-negative organisms. In dilution of 1:1,000 the mixture has a phenol coefficient for many of the pyogenic organisms. It is nontoxic to normal tissues, although it stains skin, mucous membrane, and granulation tissue (in fact, everything it comes in contact with) a purple-brown color.

Although dyes are messy to handle, this is insignificant in view of the facts that the morbidity and mortality from burns and the incidence of scar deformity have been markedly reduced with this treatment. Aldrich himself states that he does not "wish to give the impression that by merely spraying

with this dye there will be no mortality. The use of the triple dye makes treatment easier," but other aspects of the treatment, i.e., treatment of the patient, in general, cannot be neglected. He further adds that "it is not the final answer to all problems presented by a burn patient," even though the method is applicable as well to third-degree burns. Contrary to popular opinion, if no infection remains in these carbonized areas, the islands of epithelium that are spared (usually around hair follicles) are enough to grow and spread. Some mild contracture of scar and an occasional infection occurs with this method. "If a new substance is found, it will have all the properties of the triple-dye method and yet be an improvement." In other words, this new substance would have to lessen still further the possibilities of infection and scarring.

This "new substance" was introduced by Pickrell[20] in August, 1941, and is one of the amazing group of sulfon drugs, sulfadiazine (2-sulfanilamido-pyrimidine). In this author's introductory series of cases it was found to be much superior to methods previously used for treating burns.

It is a nontoxic, extremely bactericidal, analgesic substance that forms a tough, thin, pliable, and transparent eschar. No contractures resulted in any of Pickrell's cases, even when used around the eyes. It makes the patient so comfortable that 100 of the 115 cases treated were able to leave the hospital on the day of admission and be observed as out-patients. Like the triple dye method, no traumatizing scrubbing of the burned area is necessary. As the eschar is translucent, the granulization and healing can be observed directly; and it is so elastic that motion can be started immediately without cracking or disturbing the eschar in any way. None of the 115 cases developed any infection while the eschar remained in place.

The solution used is one of 3 per cent sulfadiazine in 8 per cent triethanolamine. It has a pH of 8.7; it is clear and has a faint yellow color owing to the oxidation of the sulfadiazine. This, although it does not materially affect the efficacy of the drug, may be prevented by storing it in dark glass bottles. It is odorless, has a bitter taste, and does not stain skin or clothing. It penetrates tissues and can be detected in the blood within several hours after being sprayed on a burned surface. After the eschar forms, the blood level approaches a minimum and remains level. Normal tissue is unaffected by the drug. The triethanolamine is nontoxic, as demonstrated in laboratory animals. If the blood level of sulfadiazine is kept below 15 mg. per cent (20 mg. per cent is the maximum for safety), it can cause no kidney damage. Temporary psychotic episodes and blood dyscrasias are rare with sulfadiazine, although blood counts, urinalyses, and sulfadiazine blood levels, taken at frequent intervals, are necessary to discover such rare eventualities. Other toxic manifestations as drug fever, drug rash, cyanosis, and jaundice seen in the sulfon drugs are extremely infrequent but should be watched for. However, in Pickrell's series only one case showed drug toxicity (kidney damage), even though sulfadiazine was administered orally to some patients as well as being used to treat the burn.

The treatment of burns is divided into two parts: (a) Treatment of shock (primary, secondary, or tertiary). (b) Treatment of the burn itself.

Treatment of shock should be undertaken *at once.* The most critical period for a burn case is eight to twelve hours after the injury has occurred. If the

patient has had a relatively severe burn and is not in shock, treat him as if he were. The treatment of the burn should not be begun until the shock therapy is well under way and the patient has a normal temperature, relatively normal blood pressure, a good pulse and respiratory rate, and his skin is warm and dry. Continue shock therapy while treating the burn. Wrap the patient in sterile sheets to protect the burn and *treat the shock*.

Primary shock is that stage that comes on immediately after the injury and is similar in every respect to surgical and traumatic shock. The contributory causes are: (a) The general withdrawal of blood plasma into the tissues and loss of plasma from the burned surfaces. (b) Severe pain from the exposed nerve endings.

Plenty of morphine should be given. Grain ⅓ to ½ can be administered with impunity and repeated every four hours, or as often as necessary to keep the patient comfortable. Pain itself is an excellent antidote for an overdose of this narcotic. So keep the patient free from pain.

Apply external heat in the form of heated blankets or a heat cradle. Hot-water bottles, electric pads, or anything available should be used to keep the patient warm.

Elman,[8] Black,[4] and others have definitely shown that the volume of blood during this stage is depleted by loss of plasma through the capillaries and larger vessels, and from the burned surfaces.

The administration of plasma at this time is to be strongly advised. If possible, plasma should be given to *all* severe burn cases. If this is not available, whole blood may be used, although this adds erythrocytes to an already too viscous blood. Parenteral fluids are contraindicated, for they further dilute the blood proteins and only temporarily stay in the peripheral circulation.

Rhoads[22] and his co-workers, Ivory,[16] and others, have shown that the use of adrenal cortical extract (eschatin, cortin) decreases capillary permeability and helps maintain the plasma level in the blood, thus reducing the incidence of secondary shock in severe burns.

Stimulants, such as ephedrine sulfate (grain ⅜), adrenaline (minims v-x), or other pressor substances; caffeine sodium benzoate (grains 7.5), strychnine sulfate (grain $\frac{1}{20}$), coramine (1 c.c.), metrazol (1 c.c.), may be administered parenterally if indicated. However, the above treatment should make their use unnecessary, except as a heroic measure.

The patient's head should be kept low (Trendelenburg position) to increase and maintain cerebral circulation. Due to the anoxia as a result of diminished blood volume and poor peripheral circulation, as evidenced by the high blood viscosity and low arterial blood pressure, it is recommended that oxygen be administered throughout the entire shock therapy by means of a Boothby mask. Poor oxygenation of the tissues is the prime factor in causing parenchymatous damage.

Secondary shock occurs from ten to twenty hours after the injury, but it may be delayed as much as two or three days, and is the so-called histamine shock of Cannon (absorption of tissue breakdown substances). The author believes it is merely a more extreme form of primary shock and should not occur if the patient is properly treated. It is due to increased hemoconcentration and can be treated with plasma transfusion and prevented by the use of

adrenal cortical hormone. During this stage the viscosity of the blood, which normally is 2.4 (taking water as 1.0), increases to 4.0 and 5.0.

Third type of shock is relatively uncommon and, if present, makes the prognosis very grave indeed. It is a prolongation of the second phase, with a blood viscosity so great (6.0 to 7.0) that the patient's own cells act as thrombi and emboli to cause cerebral, liver, and kidney damage (anoxia). This stage of shock is hard to treat, for fluids injected into the venous side of the circulation cannot be pumped out to thin the capillary blood. Prevention is the best treatment for this stage. Administration of plasma via the sternal marrow is the treatment of choice in this stage of shock (Tochantin).

Burns are classified as first degree (stage or erythema), second degree (erythema plus bullae formation), and third degree (stage of carbonization; erythema with bullae are also present).

In discussing the treatment of the burn itself, the general details will be taken up before going into the specific methods for the triple dye and the sulfadiazine-triethanolamine techniques.

In general, the following procedures are followed in the order in which they appear:

(a) Wrap the patient in sterile sheets and proceed to the operating room.

(b) Treat the shock; do not proceed until you feel reasonably assured that the patient is out of shock, and is being treated for shock during the burn treatment (see above).

(c) Don mask and cap. All observers, assistants, and nurses do the same. Remember outside contamination (especially droplet contamination from the noses and throats of spectators, etc.) causes infection of burns.

(d) Scrub in the usual manner; don gown and gloves (all assistants do the same).

(e) The burned areas are gently and carefully débrided of all dead tissues and bullae. The area is not cleansed or scrubbed in any way unless to remove, gently, previously applied grease or oil. Antiseptics are not applied to traumatize further the tissues.

(f) The dye or sulfadiazine solution is sprayed on the débrided area with an atomizer, as outlined below.

(g) If delayed blisters appear twenty-four to seventy-two hours or more later, the above procedure is repeated.

The triple-dye method is as follows:

(a) Follow the foregoing outline in preparing the patient. *Treat shock!*

(b) The dye solution (1 per cent aqueous solution of crystal violet and brilliant green and 0.1 per cent solution of neutral acriflavine) is sprayed on the burned areas every hour for eight hours. An eschar forms at the end of the first or second spraying, but is not complete until the end of eight hours.

(c) Keep a heat cradle (not over 90°) over the patient.

(d) Observe the eschar daily for soft, moist spots.

(e) Excise soft, moist (infected) areas, gently sop up secretions with a sterile sponge, then respray as above.

(f) Continue daily observations until epithelization is complete or the granulation tissue is built up enough to take a graft.

(g) In case of massive infections (seen where burns occur around body orifices and are continuously being contaminated), the eschar may be removed and the raw surface treated with saline spreads until clean and then resprayed.

(h) No dressings of any kind are applied.

(i) The eschar comes off in about one to three weeks with the surface beneath more or less normal skin. Rarely and only where the burn is third degree is a skin graft needed.

The sulfadiazine-triethanolamine method is as follows:

(a) Follow the same general outline for preparing the patient (see above). *Treat shock!*

(b) Spray the burned area with the solution every hour for the first twenty-four hours. A heat cradle at 90° should be used.

(c) Spray every two hours the second day.

(d) Spray every three hours the third day.

(e) Spray every four hours the fourth day. By this time a thin translucent eschar has formed that is soft and pliable.

(f) Encourage active motion; if the burn is not too extensive, allow the patient up and about. If the burn is less than 20 per cent of the body surface, discharge from the hospital. Motion prevents contractures.

(g) In about ten days the eschar begins to loosen from the epithelium beneath. Applications of the mixture or saline compresses speed the separation. In third-degree burns, allow the eschar to remain in place for three weeks. This type may need grafting. This is done after saline spreads have been applied for several days. Observing the burn through the translucent eschar makes the rare infection easy to detect. If present, soak off the eschar and respray.

(h) Ambulatory patients, i.e., those treated and sent home within twenty-four to forty-eight hours, are sprayed frequently during their stay in the hospital, and the burns are covered with sterile vaseline gauze or an ointment made up of 5 per cent sulfadiazine and 8 per cent triethanolamine. In these cases no eschar forms, so they are seen every twenty-four to forty-eight hours and the burns are resprayed and redressed with the vaseline gauze or the ointment. This is repeated until the burn has healed.

During wartime the treatment of burns presents several added problems. First, they are usually complicated with other injuries, such as fractures and soft tissue wounds. Secondly, the problem of sepsis is a greater one, because of the great amount of contamination and the inability to give the wounds and burns the immediate care needed to prevent infection. Koch believes that a wound or burn is contaminated and, therefore, potentially septic two hours after injury, provided no treatment is given the patient in that time. In the author's experience, six to eight hours are the upper limits. However, since the advent of the sulfon drugs and the "plaster-cast-let-alone" treatment, the latter being revived during the Spanish Civil War, it is possible to treat even these potentially infected wounds as "clean." Since the introduction of the sulfadiazine-triethanolamine treatment, this problem should be further simplified.

The spore-forming organisms are all too frequent contaminants of wounds in wartime; the administration of prophylactic doses of combined tetanus gas bacillus antitoxin is obligatory. Then again, as the eschar seals the burn from the outside air, a treated burn is an excellent place for the growth of these anaerobic organisms. In civilian practice some surgeons routinely give at least the tetanus antitoxin (3,000 units) to all their burn cases.

In reviewing the literature extant on the treatment of burns since the start of the present war, it is readily seen that no standardization of treatment has been devised. It is the author's impression that the greatest difficulty with this problem lies in the failure to set up a definite standard simple plan for first-aid and hospital treatment based on one or the other of the specific methods described in this paper. There are too many diversified forms of treatment, depending on the type of burn, its location, whether or not it is complicated by a wound, etc. There can be only one method for routine, standard treatment.

All the British authorities have agreed upon the necessity of treating the shock. They have reduced the mortality tremendously by using heat cradles, plenty of morphine, routine administration of adrenal cortical extract (2 c.c. per dose) in liberal amounts, and, best of all, the use of plasma. Primary shock from burns is not seen often in wartime Britain, but 80 per cent of deaths from this injury are due to secondary shock.

If the patient has a hemoglobin of over 100, it is obligatory to give him plasma and keep giving it until the hemoglobin readings reach more normal figures. Pseudopolycythemia is a good index to the increased viscosity of the blood—and taking a hemoglobin and/or red blood count are easy, quick ways of determining the concentration of blood.

In severely wounded cases British surgeons find it advisable to administer oxygen routinely by means of the K. L. B. mask (Boothby type). The Royal Navy, which has had this type of mask available for use, has found it invaluable in saving lives, especially in cases that are complicated by "blast lung."

Hot coffee (6 ounces) by rectum or by mouth has been a successful adjunct in treating the shock cases. It is an excellent stimulant and helps keep the patient warm. The latter factor is stressed, and a large percentage of injured men that were successfully kept warm at Dunkirk, lived, in spite of the lack of much else in the way of treatment. Any means available was used. Immersion in the sea seemed to help preserve body heat, for none of the burn cases rescued from the Channel were in shock.

As for the first-aid treatment there are as many methods as there are medical officers in the British Army and Navy. Tannic acid jelly seems to be the most generally used, since all tanks, gun turrets, etc., are equipped with tubes of this material. However, gentian violet, picric acid, acriflavine, paraffin, gentian violet and merthiolate, amertan jelly, tannafax, and tannax all have their advocates.

The tannic acid jelly is the favorite for first-aid applications; and when a burn case is first seen, the routine consists of plenty of morphine, keeping the patient warm, liberally spreading tannic acid jelly upon the undisturbed burn (no first-aid station débridement), and applying two or three layers of gauze over it and holding them in place by a bandage. This is left *in situ* until the patient reaches the hospital. Of course, his other wounds are treated, splints are applied, etc., at same time the burn receives attention, placing the jelly into wound surfaces if necessary.

The patients mentioned above who were immersed in sea water for long periods had a very low incidence of infection. Wakeley[24] favors the use of gentian violet in first-aid treatment over the tannic acid jelly because of its greater germicidal action, and for this reason even places this material into compound fractures.

After the patient gets to the hospital, he is assigned to a special ward for burn cases and taken to a special operating room. Due to the high incidence of crossed infections, burns are treated and bedded in "clean" operating rooms and wards.

If much dirt, grease, fuel oil, and dead tissue are present after the shock has been treated, the burn is cleansed and débrided under aseptic conditions,

the patient being anesthetized with either intravenous pentothal sodium or evipal (evipan). No untoward effects were encountered from these anesthetics. Little gas oxygen anesthesia is used and chloroform and ether are never employed.

The débridement consists of first shaving the hair and removing the fingernails and toenails, if necessary, around the injured parts which are then gently cleansed with ether soap. Cotton pledgets and soft friction are used. The wound is then rinsed; or if deep, irrigated with saline solution. The burned area is then dried with a hair drier.

The triple-dye method is used rather extensively in most British military hospitals. The incidence of infection and toxemia has been dramatically reduced by this method. The tannic acid-silver nitrate method is still used. However, where deformity is feared (i.e., in burns of the neck, face, flexor surfaces, or the hands), this treatment is avoided. The eschar of the triple-dye method, when used on the localities mentioned, is removed as soon as granulation tissue is favorable for skin grafting. This prevents deformity. Wakeley[24] uses a combined aniline dye (gentian violet) tannic acid treatment. He applies the dye first for its germicidal effect and then tans the area by the usual method.

Splints and casts are used by many surgeons to put the part to rest, but are not applied until all swelling has subsided. Heat cradles are kept over the patient at all times. Limbs are elevated to promote venous drainage and keep down swelling.

Saline immersion baths are used by some men for treating burns, especially third-degree burns and burns of the hands and flexor surfaces. Saline packs are used upon the face. This method also helps separate the eschar.

Mathews[18] uses the "tulle gras" method of treatment. This dressing is made up of close-mesh gauze and is cut into suitable squares, impregnated with soft paraffin (98 parts), balsam of Peru (1 part), and olive oil (1 part). These squares are packed in a covered metal container, each separated by white paper to facilitate removal, then the above paraffin mixture (250 Gm. for a container 5 inches square and 2 inches deep) is poured into the tray and is sterilized for an hour at 150° and the container is sealed. A local anesthetic (1 per cent decicain or 0.1 per cent percaine) may be added. This is applied to all raw surfaces and left *in situ* as long as the paraffin base lasts, and should be removed by saline immersions of the part. Some authors have used this method on hand and face burns. Gauze dressings saturated in saline are placed over the "tulle gras."

Cod-liver oil and cod-liver oil ointments are rather extensively used in Britain, especially for burns of the face and hands. German army doctors have been using this method; they use tannic acid for other burns.

Bunyan uses the "bag method" in treating his hospitalized burn cases. This consists of primary irrigation and cleansing with 10 per cent electrolytic hypochlorite solution at 100° with the patient under an anesthetic. Coated silk watertight envelopes are then placed on the limb over the burned area. No other covering is used. The envelope is so rigged and is sealed at each end that hypochlorite solution can be run through it, irrigating the wound. This is done two or three times daily.

Hudson[15] modified this method of Bunyan's by using impregnated silk as an occlusive dressing (where the bag cannot be used). If a large area of the

body is burned, he uses a large envelope that includes the entire body, then applies irrigations of hypochlorite. He also advocates the use of sulfathiazole cream in infected burns.

Extensive and third-degree burns are also treated by a large number of men by the saline-immersion method, 0.9 per cent saline at body temperature, which is thermostatically controlled. Some apply "tulle gras" after the immersions until grafting can be done.

Gas warfare has not been used to any great extent during the present war, and it is beyond the scope of the paper to discuss in detail the treatment of such burns. However, after specific decontamination, they are treated by the usual methods.

Chemotherapy is extensively used as an adjunct in treating burns. Sulfanilamide seems to be the drug of choice. Sulfapyridine is used only if pneumonia complicates the picture (this is not infrequent in severe burns), because of its "toxic reactions," such as nausea, vomiting, etc. Sulfathiazole is prescribed as well as the sulfanilamide, but not as extensively. Sulfadiazine was not mentioned by any of the authors. Powdered sulfon drugs are not used on burns, although they are put into wounds. As previously stated, Hudson[15] uses a sulfathiazole cream and Robson[24] uses a glycerin sulfonamide paste ("euglamide") in infected burns.

Plaster casts are used in deep burns or where the burns are complicated by other injuries. Casts are not applied until edema subsides. Then the limb is wrapped in sterile sheet-wadding and the part is encased in plaster in the position of function. They are not removed until healing has taken place, even though they become foul-smelling. This is the lesson learned from the recent Spanish Civil War. Apparently a bacteriophage is formed that inhibits bacterial growth in the patient's infected tissues, finally eliminating the infection. Little or nothing is mentioned, in any of the papers reviewed, about the routine administration of tetanus or gas bacillus antitoxins, nor is the incidence of these complications discussed.

In conclusion, the author can only urge that a definite, simple standard routine treatment for burns be adopted by the United States armed forces. In his estimation the sulfadiazine-triethanolamine solution is the treatment of choice. As was indicated, it can be used as an ointment in first aid and as a solution in the hospital treatment; it is stable, nontoxic, nonstaining, and highly bactericidal. The drugs can be placed into soft tissues and compound fractures or other wounds so that complicated burns need not have separate forms of treatment. It can be safely used on the face, neck, hands, and extensor surfaces with little fear of contractile scar deformity, even in third-degree burns. In short, it has all the advantages and none of the disadvantages of all the other types of treatment.

The standard routine treatment suggested is as follows:

FIRST-AID TREATMENT

(a) *Keep patient warm* by any means possible; blankets, coats, hot coffee, hot water bottles, etc. Place the patient out of the weather.

(b) *Relieve pain* by giving adequate doses of morphine, grain 1/3 to 1/2, every half hour if necessary.

(c) *Prevent loss of plasma* and plasma proteins by administering adequate amounts of adrenal cortical hormone (2 c.c. dose) parenterally. Plasma infusions should be started.

(d) *Apply sulfadiazine (5 per cent)—triethanolamine (8 per cent)—stearin ointment* ("lubricating jelly" should be used as a base to make a water-soluble ointment) to the otherwise undisturbed burned areas. No débridement is done. Apply a sterile dressing to protect the burned area.

NOTE.—In the alternate method the triple-dye ointment made up of an aqueous solution of 2 per cent crystal violet and brilliant green and a 0.2 per cent acriflavine in a "lubricating jelly" base should be used for the first-aid treatment instead of the sulfadiazine-triethanolamine ointment.

(e) *Remove the patient to a hospital* as soon as possible.

HOSPITAL TREATMENT

(a) Wrap the patient in a sterile sheet.

(b) Begin antishock therapy.

1. Place the patient in Trendelenburg position.
2. Apply heat cradle (90°).
3. Plasma should be given; adrenal cortical extract should be routine.
4. Give more morphine if indicated.
5. Oxygen should be routinely administered by means of a Boothby mask.

(c) Give the tetanus-gas bacillus antitoxin. (Is the patient sensitive to horse serum?)

(d) Operator and assistants prepare themselves for a sterile operation.

(e) The patient is draped.

(f) If burn and other injuries are extensive and dirty, requiring cleansing as well as débridement or other surgical procedures, intravenous sodium pentothal should be administered.

(g) Débridement and *careful, gentle* cleansing is performed. Wash and irrigate with sterile saline. Dry with hair dryer.

(h) A solution of 3 per cent sulfadiazine and 8 per cent triethanolamine is sprayed on the burns.

(i) The patient is placed in bed. Shock therapy is continued (plasma and cortate are administered) until blood shows normal viscosity. (Check hemoglobin and red blood cells.) A heat cradle is kept over the patient. *Be careful;* shock may be delayed up to ten and twenty hours after the initial burn.

(j) The burn is sprayed hourly the first day; every two hours the second day; every three hours the third day; and every four hours the fourth day. By this time the eschar is complete.

NOTE: If the sulfadiazine-triethanolamine solution is not available then the triple-dye aqueous solution (1 per cent crystal violet and brilliant green and 0.1 per cent acriflavine) should be used to spray the burn. The spraying is done every hour for eight hours.

(k) Check blood and urine daily for sulfadiazine levels and crystals, respectively. Do not allow the former to go above 15 mg. per cent.

(l) Observe the eschar daily. It is translucent and the tissues beneath are in clear view. If the burned surface beneath appears infected, remove the eschar with saline soaks, apply sterile saline spreads for a few days and then respray.

(m) When the eschar begins to loosen (about tenth day), warm saline immersions can be used to help loosen it. Do not remove a third-degree burn eschar under two weeks. It is best to leave it in situ for three weeks.

(n) Encourage motion. Get the patient up and about as soon as possible; on the day of admission, if feasible. The soft eschar does not interfere with motion.

(o) If large denuded areas of granulation tissue remain after the eschar falls off, apply saline spreads for a few days and perform skin graft as soon as possible to prevent disfiguring scars.

The triple-dye method can be substituted for the sulfadiazine-triethanolamine ointment and solution. It is almost as efficient, but is messy and has more tendency to cause disfiguring scars, although these are not as severe as in the tannic acid treatment. The dye method has an advantage in that it is more economical. Sulfadiazine is still a rather expensive drug; however, the added cost is justified by the lowered incidence of morbidity, mortality, and deformities. The tannic acid treatment for burns should be discarded, for with the newer treatments this method has outlasted its usefulness.

BIBLIOGRAPHY

1. Aldrich, Robert H.: Treatment of Burns With a Compound of Aniline Dyes, New England J. Med. 217: 911, 1937.
2. Aldrich, Robert H.: Treatment of Burns With a Compound of Aniline Dyes, Maine M. J. 28: 5, 1937.
3. Allen, Harvey S.: Treatment of Superficial Injuries and Burns of Hand, J. A. M. A. 116: 1370, 1941.
4. Black, D. A. K.: Treatment of Burn Shock With Plasma and Serum, Brit. M. J. 2: 693, 1940.
5. Bunyan, John: Treatment of Burns and Wounds by the Envelope Method, Brit. M. J. 2: 1, 1941.
6. Cohen, S. M.: Experience in Treatment of War Burns, Brit. M. J. 2: 251, 1940.
7. Devine, J. B.: The Three-Dye Treatment of Burns, M. J. Australia 1: 924, 1939.
8. Elman, Robert: The Therapeutic Significance of Plasma Protein Replacement in Severe Burns, J. A. M. A. 116: 213, 1941.
9. Flemming, Cecil: Combination of Burns and Wounds, Proc. Roy. Soc. Med. 34: 53, 1940.
10. Florcken, H.: The Treatment of War Burns, Chirurg. 12: 89, 1940.
11. Glover, Donald M., and Sydow, Arnold F.: Fifteen Years of the Tannic Acid Method of Burn Treatment, Am. J. Surg. 51: 601, 1941.
12. Grower, Walter A.: Treatment of Fresh Burns With Scarlet Red Bandage and Moist Sulfanilamide Dressings, J. Iowa M. Soc. 31: 234, 1941.
13. Grigsby, B. C.: Sulfanilamide in Burns, Virginia M. Monthly 67: 306, 1940.
14. Hardin, Parker C.: Cod-Liver Oil Therapy of Wounds and Burns, South. Surgeon 10: 301, 1941.
15. Hudson, R. V.: Wounds and Burns Treated by Coated Silk Fabric, Brit. M. J. 2: 7, 1941.
16. Ivory, Harry S.: Use of Cortical Adrenal Extract in Treatment of Severe Burns, Mil. Surgeon 87: 423, 1940.
17. McClure, Robert D., and Lam, Conrad R.: Problems in the Treatment of Burns; Liver Necrosis as Lethal Factor, South. Surgeon 9: 223, 1940.
18. Mathews, D. N.: Dressing Open Wounds and Burns With Tulle Gras, Lancet 1: 43, 1941.
19. Murless, B. C.: Treatment of Infected Burns, Brit. M. J. 1: 51, 1940.
20. Pickrell, K. L.: A New Treatment for Burns, Bull. Johns Hopkins Hosp. 69: 217, 1941.
21. Pierce, Wilmot F.: Use of Sulfhydryl Solution in Treatment of Burns, Preliminary Report, Am. J. Surg. 53: 434, 1941.
22. Rhoads, J. E., Wolff, W. A., and Lee, W. E.: The Use of Adrenal Cortical Extract in Treatment of Traumatic Shock of Burns, Ann. Surg. 113: 955, 1941.
23. Robson, J. M., and Wallace, A. B.: Use of Glycerin-Sulfanilamide Paste in Treatment of Burns, Brit. M. J. 1: 469, 1941.
24. Wakeley, C. P. G.: War Burns and Their Treatment, Practitioner 146: 27, 1941.
25. Wakeley, C. P. G.: The Treatment of War Burns, Surgery 10: 207, 1941.

MILITARY DISCIPLINE*

A Problem in Readjustment

Lieut. Col. Patrick S. Madigan, M.C., U. S. Army, and
Lieut. Col. Malcolm J. Farrell, M.C., U. S. Army

SINCE the birth of our nation it has been necessary to expand rapidly our military forces to meet the major military conflicts in which our country finds itself involved. Once again we are faced with the problem of raising a large military force, consistent with military efficiency and the urgency of the situation, in as short a time as possible.

In this swift expansion many persons must make a rapid readjustment; their being uprooted from civil life and placed in a military environment may, in many cases, result in neuropsychiatric difficulties. This change may alter not only a person's hopes for the future but also his manner of living substantially. Two changes are especially difficult for him: first, the loss of his individuality; and, second, the loss of personal liberty. He soon comes to feel that he is a cog in a vast machine, required to give up certain rights and privileges which he has enjoyed and to which he feels entitled. He is subjected to military discipline, and is placed among strangers. It is easily understandable that a great strain is put on his power of adjustment. He finds that more demands are made of him, more responsibility is placed on him. He is compelled to undergo certain hardships, such as participating in maneuvers, and when a state of war exists, he is faced with the possibility of death or injury.

All these new adaptations are, from their very nature, difficult for the normal man, to say nothing of the unstable one. It is obvious, then, that many men will fail completely when these new adjustments are thrown upon them, and a frank psychosis will result. Many conflicts present themselves in the nature of behavior problems and a breech of discipline, which sooner or later brings the man into conflict with military law. It is the purpose of this paper to discuss the important angle of military discipline, to explain its purpose, and thereby to help the person to effect a more healthy readjustment from civil to military life.

The subject of military discipline is little understood outside the Army and, it must be confessed, is not always viewed in its proper light even within the Army. The difficulty may lie in the fact that most people either do not, or will not, grasp the proper meaning of military discipline, its nature, its purpose, its necessity, and finally, most important of all, its spirit.

Living within an atmosphere of strict discipline from the cradle to the grave, most persons fail to realize this because only a gradual adjustment is necessary. Practically every experience through which we pass and through

*From the Neuropsychiatry Division, Office of the Surgeon General.

which we acquire knowledge is in the nature of a disciplinary correction inflicted upon us by some agency of nature or of civilization.

Outside of military circles the average man recognizes fully the necessity of discipline in his family relationships, his business, and his relations with his fellow men. This is made easy for him to understand, since he is familiar with the reasons and conditions that surround the rearing of a family and that must necessarily govern the relations of men in civilized intercourse. All this information is gathered gradually by the average man, and there is no necessity to familiarize himself with it or to make a readjustment to it rapidly.

On the other hand, the average man is not familiar with conditions in the military service or, if he has some information, it is, no doubt, based on false information and is colored by fancy or rumor. Ignorance of a situation always provokes a reaction of fear, and when a person has knowledge of a situation he tends to lose at least some of the fear. It follows then that when the man does not understand the reasons for the rules and regulations required by military discipline, he is more than likely to view them with fear and intolerance. Even within military circles, among men who should be familiar with the reason and spirit of military regulations and discipline, many make the serious mistake of confusing the exercise of authority with the maintenance of discipline. In brief, they appear to think that discipline must be maintained wholly for the purpose of upholding their authority; and again, both outside and inside military circles, many fail to understand the spirit upon which true military discipline is based and must be based. Failing in this, they fail altogether, for the simple reason that men can understand the principle of military discipline only when they appreciate the underlying spirit.

Without the proper spirit there can be no such thing as discipline in any army. Proper discipline should, in no sense, be based upon the fear of disciplinary correction, since in this instance we have merely the blind obedience of the schoolroom type of discipline. The discipline on which a successful army must be built is a different kind; a kind that endures even when every semblance of authority has vanished, when the leader of the team has fallen, when members of the team are dropping out one by one, and when the only driving power that remains is a strong and unconquerable spirit of attainment. This concept gives us at once a working definition of military discipline: the spirit of the team.

Oftentimes the question is asked, "Of what necessity is military discipline?" The answer is readily found in the necessity for teamwork in any understaking, irrespective of whether it be in business, on the baseball or football field, or in the training of a vast army.

One element of good discipline is the fear of losing the respect of one's fellow members of the team, whether he be officer or soldier. This desire for esteem is one of the essentials of good military discipline. It is to be seen in all trained and disciplined units, the members of which feel for each other a natural respect and admiration. The knowledge that he enjoys the respect and admiration of his fellow soldiers is a source of the greatest pride to each member of the unit. The desire to retain this respect, to be looked upon as a worthy member of the unit, will greatly lessen the fear of injury.

In our present emergency we are attempting to inculcate in the minds of our soldiers the necessity of protecting our country, of taking the offense, and of bringing the war to a successful conclusion as rapidly as possible. Ideals have been set up, and the soldier must be helped to realize that he is fighting for a cause. He must be aided in finding satisfaction in serving such a cause and in expending his energy for that cause. It is needless to point out that Pearl Harbor and the subsequent events have given us the cause. The soldier must be physically developed, trained to conserve health, and he must learn to perform with technical skill his part in every incident as a member of the great team of which he is a member.

Besides these qualifications he must have the mental attitude of a soldier. To attain this proper attitude is an important step; there is an ideal characterized by the tendency to correct action and supreme satisfaction in such action.

In a short treatise Pew* places considerable stress upon the process of acquiring proper military habits and gives four maxims:

1. Select the habit.
2. Demonstrate the habit.
3. Secure abundant and genuine practice, with every effort of will and attention directed toward acquiring the habit.
4. Allow no exceptions.

Under this grouping this author classifies military bearing, courtesy, putting forth physical and mental efforts at the highest possible level, mental and physical self-control, neatness and order, smartness, exactness, and promptness; subconscious obedience, mental alertness, and confidence; and last but not least, teamwork in all military efforts. The old maxim "A chain is as strong as its weakest link" applies more than ever to the military forces of this era. Likewise, the same maxim applies individually to both officers and enlisted men.

It is held by many civilians and civilian agencies that military discipline would be desirable for those men who have failed to make a satisfactory adjustment to civilian life. It is to be pointed out that the Army is one of the elements of national defense, and its present mission is one of preparation for offensive and defensive warfare. To forge into that great team links that are defective or weak is to invite disaster. The Army is in no sense a social service or a curative agency. It should not be considered a haven of rest for the wanderer or shiftless, or a corrective school for the misfits, the ne'er-do-wells, or the chronic offender. Furthermore, it is neither a gymnasium for the training and development of the undernourished or underdeveloped, nor is it a psychiatric clinic for the proper adjustment of adults who are in need of emotional development and guidance. The man entering the Army will have to assume one of the greatest of obligations: that of protecting his country and its ideals. Therefore, there is no place in the Army for the physical or mental weakling, the potential or present behavior problem. If a man is a behavior problem in the community, he will certainly become a more intensified one in the Army.

It is to be further stressed that many men with abnormal personality traits are capable of satisfactory adjustment in civilian life. For this class there

*Pew, W. A., Maj. Gen., M. N. G.: *Making a Soldier*, 1917.

are in civilian life numerous avenues of escape which are closed to them when they enter the great team of the Army. Necessarily, teamwork of any type, more especially the Army, requires that a man readjust himself to a more or less inflexible and limited environment. When thrown upon their own meager resources of adaptation to Army life, requiring contact with all types of personalities, with limited and necessarily circumscribed opportunities for self-expression, many who are just able to adapt themselves under the most favorable conditions will not fit into that one iron mold which experience has taught is essential to military success. It is well to point out that over 50 per cent of the present beneficiaries of the Veterans' Administration are men of this type who were accepted in the Army during World War I.

The average selectee coming from the average American home has received the care, protection, and indulgence of fond parents and friends. They have made an effort to protect him from disturbing and disrupting influences that might interfere with his normal development. His parents have been interested to see that he wore the proper clothing and had the protection for all types of weather. New problems were taken over by his protectors who either solved them for him or helped and advised him in his own solutions. He has been guarded, protected, and steered, while his personal responsibilities have been at a minimum. He has not been trained to accept life as it really is.

Our soldiers come from all walks of life—from distant points in our country, from farm and factory, from colleges and benches; the rich and the poor, the educated and the illiterate—all are thrown together into a heterogeneous mixture subjected to the same military discipline, the same routine, and the same regulations. The soldier is called upon to recognize, appreciate, and conform to the wishes of others about him. He must live in close contact with his fellow soldiers and adjust himself to their method of living.

The transition from civil to military life will be made much easier for him if he understands the necessity for military discipline, the spirit of the team, and the real meaning of it. Americans are well adapted by their education and recreational experiences to realize the importance of teamwork, but sometimes it is necessary to point out to them that military discipline is a preparation and is necessary for the development of good teamwork.

It is astonishing how well and how rapidly recruits adapt themselves, even under the most difficult situations. It is the duty of each officer to keep these situations in the foreground and to take every opportunity to advise and counsel the men placed under him for proper training in military life. Each officer should regard himself as a member of a team in which each of his soldiers is an important member. Thus is built up a chain forged of confidence and respect one for the other, which will provide a pillar of mutual strength, both mentally and physically, when times of stress are greatly increased. It is, then, through military discipline that a strong, efficient team with high morale can be developed that will satisfactorily achieve the goal set for it. By understanding the reason for it, the man is enabled to make the transition from civil to military life in a more satisfactory manner.

WAR NEUROSES*

MAJOR NORMAN Q. BRILL, M.C., U. S. ARMY

INTRODUCTION

THE subject of war neuroses met with little interest and came in for little discussion until the World War. The subject became a vital one and has continued to demand attention ever since. It was during the war that for the first time such large numbers of individuals suffering from nervous disorders confronted the public, physicians, and government agencies. Psychiatry, a field of medicine which to a great extent was practiced in crowded institutions behind locked doors, was forced into the open and into its lap was thrust a problem which it has not yet fully solved. And now again it is to be given a task that will keep it occupied for many years to come. It is, therefore, important that physicians in the armed services and others who have occasion to deal with the psychiatric patient of preparedness and of war be familiar with the present-day concept of war neuroses.

DEFINITION AND MECHANISMS: IMPORTANCE OF PREDISPOSITION

Since war now embraces civilian as well as armed forces, the term will now necessarily be applied to both groups. Under war neuroses have been included a great many varieties of conditions. The diagnosis of exhaustion neurosis, shell shock, concussion neurosis, fright neurosis, and neurocirculatory asthenia have been applied to the manifestations which were so prevalent in soldiers during the World War. The symptoms were varied and consisted of tremors, inability to stand or walk, blindness, paralysis, stammering, and loss of voice. Many soldiers suffered from nightly frightening dreams and awakened with intense anxiety. Others complained of marked weakness with fatigue brought on by the slightest exertion, palpitation of the heart, or of dizziness or faintness, headache, or pains referable to practically every part of the body.[1]

The occurrence of such large numbers of these cases suggested a specific relationship between these conditions and war, and led to the naming of these conditions war neuroses. Referring to the World War, one authority stated that it is noteworthy that since the war and in the decade or two before it, such gross hysterical phenomena were only rarely met with in civil practice.[1]

However, it was observed that many soldiers developed the symptoms after slight or no injury during combat, behind the lines, in concentration camps away from the front, and in training camps at home. Also, the war neuroses differed little in their symptomatology from those of civil practice.[1]

The London correspondent of the *Journal of the American Medical Association* wrote the following in February, 1940:[2] "During the last war, the

*From the Neuropsychiatric Section, Station Hospital, Fort Bragg, North Carolina. Reprinted from The Military Surgeon 90: 390 (April), 1942.

large number of neuroses engendered by fear, shock, physical and mental strain or lack of sleep caught the medical officers of the army unprepared with any organization or agreed method of treatment. These neuroses were popularly called 'shell shock,' for it was supposed that they were due to the physical effects of high explosives. But it was found that similar symptoms occurred among men who had never been exposed to explosives and were well known in civil life. Moreover, many men who were blown up or buried beneath debris to the same extent returned to duty without suffering any permanent ill effects. The minister of pensions convened a conference under the chairmanship of Lord Horder to advise the government as to the general principles for dealing with these cases. Its report has just been presented and issued to the medical services." The report points out that most of the neuroses took the form of either an anxiety state or hysteria. The anxiety in all the cases was far out of proportion to the cause.

"In 1920 the army council appointed a committee to collate the medical experience of the war for future use. The committee reported: 1. The term 'shell shock' has been a gross and costly misnomer and should be eliminated. It is a catchword which reacts unfavorably on the patient and others. 2. The war produced no new nervous disorders. Only 5 to 10% of the cases that were called shell shock suffered from genuine concussion—while 80% consisted of 'emotional shock'—either acute in men with neuropathic disposition—or developing slowly as a result of prolonged strain and terrifying experience, the final breakdown being sometimes due to some trivial cause. In differentiating cases of concussion from those with emotional shock, it is well to remember that though patients with emotional shock may say that they became unconscious, careful questioning will show that they can recall most of their experiences."

Many cases now in training in Army camps are entering hospitals with nervous disorders. During wartime these cases might be called shell shock, if they started during a bombardment and were seen by physicians who were not familiar with the true nature of the condition.

In a vast majority of cases of neuroses that are being admitted to the hospital at Fort Bragg, a history of long-standing nervousness can be obtained. Many of these individuals frankly admit that they are no worse for being in the Army—that they "have always been that way." No statistics are available at present, but it is rare to find an individual whose nervous trouble began after enlistment or induction into the Army.

If the reaction, then, is not specific for war, it becomes necessary to explain the appearance of such large numbers of cases. It is important to consider first the question of predisposition to neurotic disorders. Individuals vary markedly in their abilities to tolerate difficult situations, as they do in adjusting to new situations. Some always worry while others are not fazed by the greatest tragedy. The "adjustment threshold" varies markedly in different individuals. It is understandable that a hypersensitive, excessively irritable, impulsive, self-centered, infantile and moody person can "take less" than a well-adjusted mature individual and will decompensate sooner when exposed to the same stresses and strains.

"In our hastily formed Army, especially under the draft system, there is a great demand on each soldier for rapid and violent adjustment. Men without any previous military experience, drawn from every conceivable walk of life, are thrown together and subjected to the same discipline, regulations and routine. It is astounding how well and how rapidly many will adapt themselves. However, many others will do poorly and hysterical reactions, psychoses and full guard houses are more than dead weight about the neck of an armed force."[8]

In *Circular Letter No. 19* of the Surgeon General's Office it states: "Not all persons are adaptable to restrictions and inhibitions of personal desires and comforts, to deprivation of rest, food, or shelter, or to the extraordinary demands for prolonged physical and mental activity often imposed by active military service. It must be remembered that many persons with abnormal personality traits who are capable of satisfactory adult adjustment in civilian life, in which numerous avenues of escape are available, will be a total loss to adjust themselves to a pattern which is more or less inflexible and, of necessity, delimited and circumscribed as to self-expression. When thrown on their own meager resources of adaptation in a military environment, in contact with all kinds of personalities, some who are just able to adapt themselves to life under the most favorable conditions, will not fit into the one iron mold which experience has taught is essential to military success."[3]

The symptoms which the patients develop under stress of training or combat are unconscious. They serve a purpose and are defense reactions to danger. The vast majority were not malingerers. Just because these soldiers had a sense of duty, esprit de corps, and of patriotism and could not honorably or safely run away from combat, they developed the unconscious conflicts and consequent symptom formation. The symptoms are for the most part hysterical. They are the result of a conflict between the instinct of self-preservation and the ideas of duty and self-respect. They satisfy the patient's ethical requirements and provide an escape from duty on the ground of physical incapacity.[1]

Man like other animals possesses instincts among which are the reactions to danger. He may stand and use his instinctual mechanism of defense to preserve himself—or flee from danger, not only to seek cover but to create as wide an interval as possible between himself and the instruments that seek to destroy him. The act of warfare meets these biological requirements of defense up to a point where there are trenches and Maginot Lines and organized retreat in the face of overwhelming odds. But the soldier cannot flee before the enemy just because he himself is in danger. He is no longer an individual, but an integral part of a battalion or company. The necessity for courage, loyalty, and self-denial were impressed upon him. Automatic obedience and strict discipline are instilled so that he will react to orders and not to impulse or instinct.[4]

However, "the individual soldier comes into a war with his own personal peculiarities. He comes with his own system of controls which have already in civilian life canalized his instincts along social channels. Further back in his childhood, his infantile aggressiveness and other instincts were subjected to a variety of controlling influences which modified them in such a way that his instincts and biological needs are only satisfied along prescribed socially acceptable

lines. . . . But however much this controlling part of the self is accepted, a perpetual state of strain is present deep in the nature of the mind, so that given sufficient provocation these submerged and now only partly statisfied instincts seek opportunity and find opportunity for operating."

"Frequently in examining the deeper psychology of the neurotic patient of the war, one found many men who stuck patiently to their duties, but who would, after a time, as the result of repeated provocation, break down under the strain. The controlling force, the better self, was no longer able to stand up against the insistent demands of an instinct which demanded either an escape from danger or an aggressive outburst. Powerful conflicts were produced between the instincts on one hand and the controlling force on the other. Where the natural self could find a way out, the soldier was able to solve his conflict by a variety of means, sometimes not laudable from the standpoint of morale . . . by reporting sick, getting around a noncommissioned officer for a soft job, or even courting the prospect of a slight wound which would allow a man to return home with mental peace and the semblance of honor. But this act could not easily be practiced and instead, when the bursting points were reached, hysterical reactions appeared. They were nature's way of obtaining some measure of adaptation, an adaptation which is in the nature of a compromise between instinct on one hand and conscience on the other."

"In cases of hysteria which had lasted sometime and were not cured immediately by the rough justice of disciplinary action or the tactful action of a wise medical officer, the reaction to war danger and stress became linked up with other conflicts in the mind which existed before the period of war service. No soldier came into the war with his mind a clean slate; it had already been written over with the story of his past experiences and conflicts. Former dangers and conditions of insecurity had either been met or had set up difficulties. Conscience had already made the coward and the courageous. The relatively mild analogues of war conflicts had already existed in civilian life. (There had been stresses and strains and causes for anxiety before.) It, therefore, did not take much time for the neurotic reactions to the war to weave themselves into the pre-existing neurotic disposition which had grown up in peacetime. It was for this reason that Emanuel Miller came to the conclusion that the cure of the hysteria of the war became in a large measure the resolution of mental problems of peacetime. Many patients with wartime neurosis, particularly those who broke down early, were found on deep analysis to be associated with long-standing feelings of insecurity. Such men were found to have spent their lives either in sheltered places or had old family ties which had thrown the responsibility for security not upon them, but upon their parents. . . . To them the establishment of new ties was difficult. Strangers could not be tolerated, new surroundings could not be assimilated. They felt themselves like strangers in a strange land and in a strange land full of dangers."

"One of the peculiar aspects of human psychology, discovered through the work of Freud, is the depth to which a man can really love himself. This love of self is not merely an expression of the self-preservation instinct, although this enters into it, but a primitive, childish, self-esteem which cannot be wounded.

Persons in whom this trait is strongly developed succeed in life admirably when they receive praise and become the centers of attention. They are not only pathetically preoccupied with their own achievements, but are sometimes even satisfied with their own selves as audience. They are proud of their bodies . . . and are literally in love with themselves. In warfare, such characters find it difficult to fit in with the group life of a regiment unless it affords them opportunity for self-expression; and when they are courageous, as they may be, it takes the form of seeking for decorations and applause. Such men, however, are terrified at the prospect of a wound, and the possibility of injury rules out all idealism. They are not the individuals who would face death for a cause. This type is prone to hysterical manifestations, to fugues and sometimes to major mental disorders. They cannot face the horrors of war, not because of sympathy for the slain, but because they identify themselves with every mutilation and death which the soldier has to witness. . . . Stated simply then, it is the conflict between conscience and instinct that can be regarded as the central motive around which all the clinical manifestations seem to collect."[4]

A clinical survey of the first group of invalid soldiers to be returned to Australia from abroad reveals some interesting facts.[5] Of 208 cases studied, 61 were due to diseases of, or injury to, the nervous system. A great majority of the patients suffering from anxiety states gave a history of previous nervousness or breakdowns. Careful inquiry into the past histories of these patients showed that they were capable of "carrying on" in civil life in spite of minor breakdowns; but the strain of military life in active service quickly revealed their mental instability. A large number might have been excluded from the Army if a truthful history had been given in the first place.

Captain John M. Caldwell, of the U. S. Army, reported the results of an intensive study of 100 American soldiers who developed psychoses during peacetime.[6] His conclusions were "the cause of schizophrenic psychoses in the United States Army is not found in any peculiar or particular demands of the service. However, the change from a familiar to an unfamiliar environment with added stresses and strains, probably plays a part in bringing latent trends to the surface. Abnormal sexual drives to a large degree are associated with the development of the psychoses and probably act as precipitating factors."

A report was published of 200 consecutive admissions to the Christian Street Hospital in Canada, in the year period from October, 1939, to September, 1940.[7] The cases included constitutional psychopathic inadequacy, mental defectives, epileptics, psychotics and psychoneurotics, alcoholics and malingerers.

Environmental and hereditary factors were investigated. In over 50 per cent the physical and mental health and economic status of the families were classified as poor or bad. Only 68 per cent showed both parents alive when the patient reached the age of 14. In eleven cases, parents were separated, seven were brought up in an orphanage, two had been adopted, and two were illegitimate.

With regard to education, thirty-two were illiterate and one hundred did not pass examinations on leaving public school. Most of these left school for no good reason. Sixty per cent had no trade; 51 per cent have had long periods of

unemployment, no settled occupation or required assistance from the family or authorities; 17 per cent had been in difficulty with the police. This included arrests for vagrancy, drunkenness, theft, and assault. Twenty-two cases were backward and eight had attended special school for backward children. Eighteen gave a definite history of a previous psychotic episode, seven of whom had actually been in a mental institution. Thirty-five had required attention for neurotic disorders prior to enlistment.

It was felt that the unsuitability should have been obvious in a majority of these patients on enlistment or in the primary training period. Often it had been recognized that the man would not make an efficient soldier and nothing was done about it. They were sidetracked on regimental duties, fatigues, etc.

In only twenty-six were strain, change of surroundings, alteration of diet, separation from families, etc., considered significant with regard to appearance of symptoms. None of those was definitely associated with war strain.

IMMENSITY OF THE PROBLEM

Even before our nation entered the war in 1917, many warnings were sounded by the French and British concerning the grave seriousness of neuropsychiatric disorders in the armed forces of that day.[4] The seriousness was emphatically brought home by a report finally rendered by the chief of the division of neurology and psychiatry of the United States Army on September 5, 1918: "Everything seems to point to our soldiers developing neuroses to a degree even greater than has occurred among the British. . . . The conditions of American life have been such that a young man suddenly taken from his surroundings where he more or less always had his own way, where obedience was never necessary, where he was taught that he was the equal of everyone, suddenly taken from surroundings of that character and forced to obedience, forced to face all this war . . . it would not be surprising if he showed his reaction to the change by developing a neurosis. . . . French neurologists have spoken of the excessive nervousness of American soldiers who have been under their care."[8b]

It developed that "whereas mental illness had been almost wholly ignored and the medical advances before the war dealt almost exclusively with physical diseases, the wide prevalence of the neuroses among soldiers was apparently leading to a revision of the medical and popular attitude toward mental and functional nervous diseases, and stimulating widespread interest in their observation and study."[8a]

"Many things were learned quickly in the war of 1914 to 1918. Although many physical disabilities could be waived, and although many of them disappeared with treatment or favorable conditions of military training, it was usually the opposite in cases of nervous or mental diseases. Such men were only too often singled out only after a considerable period of training, during which time they received pay, equipment and maintenance, wasted the time of instructors, interfered with training and filled hospital beds urgently needed for others. Many of them would have been better off to all concerned, if not even of material assistance to their country, had they been left in their accustomed

surroundings. If they became soldiers, they were almost certain to present eventually a serious economic problem to the nation in the form of government hospitalization and care."[8d]

"Neuropsychiatry was a new field in the war of 1914 to 1918. It interfered with established military routine and order and no doubt had a struggle in hastily orienting itself in this new situation. Officers complained that if specialists did not cease eliminating the unfit, there would be no Army left. It was felt that training which transformed poor physical specimens into robust fighters could do the same for those who had nervous or mental disabilities. As a result, recommendations for rejection or discharge were often waived by line officers. Eventually it was discovered that the nervous and mentally unfit were greatly embarrassing the American Expeditionary Forces. On July 15, 1918, General Pershing urgently cabled the Chief of Staff that more intensive effort be made to eliminate such men. After this, rejections and discharges were carried through with less difficulty."[8c]

"Although many conditions were clear-cut and sufficient for outright rejection or discharge, others were more difficult. These proved to be constant sources of annoyance and trouble to the officers, forming the larger number of the absentees, the discontented, the inefficients, the inmates of the guard house, and the frequenters of the regimental infirmary. These were the cases which complained of being dizzy, faint and bewildered at critical moments."[8d]

"It was said that no other class of men made for so much mischief in the United States Army as did the feeble-minded. Their records prove that physical health, strength and ability to get along in civil life did not, in themselves, insure satisfactory Army service. The strange and exacting environment of the Army was too much for them."[8d]

"The need for routine neuropsychiatric examinations of commissioned officers was expressed."[8e] "A startling number of defects was found among those attending officers' training camps, and it was felt that if all candidates for commissions had been subjected to a thorough neuropsychiatric examination, many would have been rejected with advantage to the military service. In fact, this failure was said to be probably the outstanding defect of the neuropsychiatric service."[8f]

"That the initial examination was important was demonstrated by the fact that 27,836 neuropsychiatric cases, or 40% of the total reported, were discovered during the preliminary examinations of recruits and draftees."[8f]

"The United States Navy, too, was not without its difficulties. The psychopath received particular attention. Quoted newspaper stories demonstrated how undesirables were urged to enter the armed forces. For instance:

"Dec. 16. Thief dismissed so that he might join the Army. D. F. pleaded guilty here today on a charge of petty larceny . . . but an indictment against him charging forgery . . . was dismissed so he might enlist in the . . . United States Army."[8g]

"In a survey of inmates of naval prisons, it was concluded that 54% are men who clearly should have been eliminated at the recruiting or training station."[8h] "Another naval writer's experience points to the fact that the psycho-

path has more difficulty adjusting himself to the service than has any other type of questionable individual. A recruit in this classification is not amenable to discipline and he ordinarily cannot comfortably be assimilated in any part of the organization without having an influence on morale."[8i]

"Eventually it evolved that from April, 1917, to December, 1919, 95,577 officers and men (both Army and Navy) were admitted to hospitals for mental disease in this country and abroad, and 41,976 were discharged for disability incidental to mental disorders in the same period."[8j] "Also of 54,117 ex-members of military service hospitalized by the Veterans' Administration, approximately 60 per cent were hospitalized because of neuropsychiatric disabilities, and cost the government almost $1,000,000,000 in the past fifteen years."[8k] "Even with the exclusion of paresis, mental deficiency, psychopathic personality and chronic alcoholism, two out of five veterans receive pensions because of neuropsychiatric disability."[8l] "Three of seven men discharged for disability from the Army in 1939 were suffering from mental disease, and suicides ranked second as major cause of death in the Army for that year."[8]

Similarly in the British Army, statistics from the last war showed that one-third of the unwounded, and one-seventh of those discharged were permanently unfit because of functional neuroses and mental disorders.[9]

TREATMENT

With regard to treatment of patients suffering from war neuroses in the field, the first essential when the patients arrive at an aid post is to convince them that they have received no serious injury. When confusion, excitement, loss of memory and disorientation are the chief symptoms, rest, warmth, hot drinks with plenty of sugar, and a dose of bromide or phenobarbital will be necessary. Restless or excited patients may be given a hypodermic injection of morphine or soluble phenobarbital combined with hyoscine. When hysterical symptoms predominate, an attempt should be made to remove them by suggestion.[2]

According to one English Army physician (Dillon), during the World War 63 per cent of the patients suffering from so-called acute anxiety states were able to return to duty after treatment which consisted primarily of rest and sleep, and relapses occurred in only 5 per cent.[9]

In cases that do not respond quickly, the prognoses are not as good and more intensive and prolonged psychotherapy is necessary. Such procedures as hypnosis, suggestion under narcosis, suggestive physiotherapeutic measures, and psychoanalysis have been used by various authors with reported good success. Many patients will, no matter how treated, become chronic invalids and require government care for the rest of their lives.

The major factor in the prevention of war neuroses is the detection and early rejection or elimination of the emotionally unstable from active combat service. This can be done if there is a greater awareness of the problem in medical circles. Physicians on induction boards should have a psychiatric as well as physical orientation and approach. Any lack of psychiatric awareness that does exist is but a reflection of the state of affairs in the medical profession in general.

However, the lesson of the World War was well learned, and it is significant that the Surgeon General has requested that medical officers examining applicants for the Army be especially alert to detect all those with any mental or nervous abnormalities who later may disrupt discipline and morale, retard progressive military training, occupy hospital beds urgently needed for acutely ill patients and finally become an economic burden to the government.[3]

If greater attention were paid to the following statement of the Surgeon General, this immense problem would in great part be solved: "The Army is one of the elements of national defense, and its present mission is one of preparation for an offensive-defensive type of warfare. It is in no sense a social service or a curative agency. It is to be considered neither a haven of rest for wanderers nor a corrective school for misfits, ne'er-do-wells, feebleminded persons or chronic offenders. Furthermore, it is neither a gymnasium for the training and development of the undernourished or undeveloped nor a psychiatric clinic for proper adjustment to adult emotional development. Therefore, there is no place within the Army for physical or mental weaklings, potentially psychotic or prepsychotic persons or behavior problems. Men who present behavior problems in the civilian community will certainly present intensified problems in the service."[3]

NEUROSIS IN CIVILIAN POPULATIONS

With regard to the problem of war neuroses in civilian populations, the general experience in Britain so far bears out the report of a conference of medical men convened before the active stage of war began. They stated that an exaggerated estimate had been formed of the liability of war to produce nervous breakdowns. This has received substantiation since the intensified air raids on Britain have been in progress, for few nervous breakdowns have occurred in spite of the suffering and material loss.[10]

The best remedy against panic and disruption for civilian populations lies in the active participation of as large a section of the population as possible. A lesson is to be learned from the Germans, who state there are no shoemakers in Germany any more, they are all fighters on the shoemaking front. Democracy too often leads to an attitude of what do I get, with a disregard of what can I give.[11]

Aggressive impulses of the people should be mobilized along certain definite channels. Each individual as far as possible should be given an active part to play. Training in first aid, instruction in modern methods of warfare, the nature and effects of high explosives and gases should not be kept as a mystery from the average citizen. He should also be taught the importance of the so-called fifth column in modern war and what measures are necessary to counteract it. Another error has been an excessive concentration of the public mind on safety first. There has been far too much talk of where to hide and how to protect one's own skin, of keeping up the morale of the nation, and of preventing panic. There has been too little talk of how to defeat the enemy. People were getting the feeling that one had only to sit and wait long enough for the enemy to be defeated without personal danger. This was the philosophy of the Maginot Line and it was a psychological disaster. It led to the feeling that we had only to produce enough machines, enough concrete and enough airplanes and to pour out enough money to win the war.[11]

REFERENCES

1. Wechsler, I. S.: Textbook of Clinical Neurology, Philadelphia, 1931, W. B. Saunders Co.
2. Foreign Letters: London, War Neuroses, J. A. M. A. 114: 424 (Feb. 3), 1940.
3. Neuropsychiatric Examination of Applicants for Voluntary Enlistment and Selectees for Induction, Circular Letter No. 19, Surgeon General's Office, J. A. M. A. 116: 2509 (May 31), 1941.
4. Miller, Emanuel: The Neuroses in War, New York, 1940, The Macmillan Co.
5. Foreign Letters. Australia, Causes of Invalidism in Military Service, J. A. M. A. 116: 2610 (June 7), 1941.
6. Caldwell, John M., Jr.: Schizophrenic Psychoses, Am. J. Psychiat. 97: 1061 (May), 1941.
7. Baillie, W.: A Summary of 200 Neurological and Psychiatric Admissions From the Canadian Army Service Forces, Am. J. Psychiat. 97: 753 (Jan.), 1941.
8. Quoted by
   Aita, J. A.: The Problem of Neurologic and Psychiatric Examination During Military Mobilization. I. General Considerations, Proc. Staff Meet., Mayo Clin. 16: 283 (April 30), 1941.
   a. Bailey, Pearce, Williams, F. E., and Komora, F. O.: Organization. In: The Medical Department of the United States Army in the World War; Neuropsychiatry, Washington, D. C., 1929, U. S. Government Printing Office, Vol. 10, pp. 5-22.
   b. Salmon, T. W.: General View of Neuropsychiatric Activities. In: The Medical Department of the United States Army in the World War; Neuropsychiatry, Washington, D. C., 1929, U. S. Government Printing Office, Vol. 10, pp. 273-302.
   c. Bailey, Pearce: Provisions for Care of Mental and Nervous Cases. In: The Medical Department of the United States Army in the World War; Neuropsychiatry, Washington, D. C., 1929, U. S. Government Printing Office, Vol. 10, pp. 39-55.
   d. Bailey, Pearce: Detection and Elimination of Individuals With Nervous or Mental Diseases. In: The Medical Department of the United States Army in the World War; Neuropsychiatry, Washington, D. C., 1929, U. S. Government Printing Office, Vol. 10, pp. 57-86.
   e. Bailey, Pearce, and Haber, Roy: Occurrence of Neuropsychiatric Diseases in the Army. In: The Medical Department of the United States Army in the World War; Neuropsychiatry, Washington, D. C., 1929, U. S. Government Printing Office, Vol. 10, pp. 151-156.
   f. Bailey, Pearce, and Haber, Roy: Analysis of Special Neuropsychiatric Reports. In: The Medical Department of the United States Army in the World War; Neuropsychiatry, Washington, D. C., 1929, U. S. Government Printing Office, Vol. 10, pp. 157-269.
   g. Stearns, A. W.: The History as a Means of Detecting the Undesirable Candidate for Enlistment With Special Reference to Military Delinquents, U. S. Navy M. Bull. 12: 413 (July), 1918.
   h. Jacoby, A. L.: Psychiatric Material in a Naval Prison at Portsmouth, N. H., U. S. Navy M. Bull. 12: 406 (July), 1918.
   i. Sullivan, H. S.: Psychiatry and National Defense, U. S. Navy M. Bull. 37: 273, (April), 1939.
   j. Strickland, B. A.: The Importance of Psychiatry in Military Medicine, The Military Surgeon 83: 83 (July), 1938.
   k. Winslow, C. E. A.: The Strength of the Nation, McCall's Magazine 68: 18 (April), 1941.
   l. Baganz, C. H.: The Importance of a Proper Psychiatric Survey in the Enrollment of Personnel of Military Forces, The Military Surgeon 86: 471 (May), 1940.
9. Dillon, F.: Neuroses Among Combatant Troops in the Great War, Brit. M. J. 2: 63 (July 8), 1939. Abstracted in Arch. Neurol. & Psychiat. 45: 168 (Jan.), 1941.
10. Langdon, Brown W.: Mental Health in War Time, Nature 147: 193 (Feb. 13), 1941. Abstracted in J. A. M. A. 116: 1774 (April 19), 1941.
11. Rogerson, C. H.: Correspondence, Am. J. Psychiat. 97: 969 (Jan.), 1941.

WAR NEUROSES. EXPERIENCES OF 1914-1918*

Lessons for the Current Emergency

Douglas A. Thom, M.D., Boston, Mass.

IT IS difficult to discuss the war neuroses as they occur during active service without thinking in terms of what precedes active service, and although you have undoubtedly heard a good deal about that phase of military activity, it may be necessary to repeat a bit of it in order to get oriented. I think we can say that the last war contributed more to psychiatry than psychiatry did to the war. I mean that the last war gave psychiatry a certain professional standing which has continued, and after the war it was found that various foundations and organizations recognized psychiatry as a branch of medicine worthy of support. I said recently that the problem of war neuroses is a three-dimensional problem. Its length reaches from war to war; the neuropsychiatric casualties of the last war still remain on our hands, and the effect of the current conflict already begins to reveal the unstable personalities in our civilian population and bring new casualties. The neuropsychiatric casualties of the two wars are overlapping. We still have some 68,000 neuropsychiatric disabilities in the government hospitals from the last war. The report of the administrator of Veterans' affairs indicates that they represent 58 per cent of all patients being cared for by Veterans' Hospitals. The economic aspect of the problem is appalling when one considers the money spent on treatment, training, and compensation. It can be said that the war neuroses begin early. They are long drawn out, they are difficult to treat, and they tend to increase for at least twenty-five years after the cessation of hostilities. It has been estimated that it costs $30,000 to care for a psychiatric disability from the time the individual breaks down until his death. We have some twenty-eight hospitals in this country caring for these 68,000 patients.

The provisions for detecting these potential neuropsychiatric disabilities before they get into service are shockingly limited and inadequate. The Government will employ a few psychiatrists at a rate of $15.00 a day and is expecting them to examine anywhere from 150 to 200 patients a day. I have pointed out to the administration that if one psychiatrist picked out but one potential psychotic every six and one-half years, he would save his salary. There is a pressing need for a careful psychiatric examination before induction of men into the military service.

The neuropsychiatric disabilities resulting from active military service represent what we call the war neuroses, and what the British used to call "shell shock." The latter term represented the viewpoint of British pathol-

*Lecture XII, reprinted from *Collected Lectures of the Metropolitan State Hospital*, Waltham, Mass., 1942.

ogist Colonel Mott at the time of the last war, who felt that "shell shock" was an organic condition produced by minute, petechial cerebral hemorrhages. The role of psychological factors in "shell shock" was minimized. It was, however, recognized eventually that a great many of the soldiers diagnosed as "shell shock" casualties had not been anywhere near a shell. This group of "shell shock" cases proved to be made up of most heterogeneous neuropsychiatric disorders, so varied that it would be of little value to classify them. In order of their frequency the neuropsychiatric disabilities of the last war were neurasthenia, conversion hysterias, anxiety states, and psychoses. We did see some mentally defectives, but they represented a small group, for about 30 per cent of all the individuals eliminated before they went into service were disqualified because of mental deficiency. There were nearly 70,000 cases disqualified as constitutionally unstable individuals, potential psychotics, epileptics, and feeble-minded.

The neuropsychiatric casualties must be considered in relation to three phases of the state of war: the period of mobilization, the period of active service, and the period of demobilization. It was recognized and the point was stressed during war activities and later in civil life that there are limits to the amount of physical and mental stress that any individual can withstand without breaking down in his adaptation to life. These limits are not static; they vary from time to time in the same individual, and they vary, of course, from one individual to another. I happened to be at the Boston Psychopathic Hospital when the last war broke out, and we had many individuals coming in immediately after war was declared. Their delusions and hallucinations were colored by the war activities, colored by what they were reading in the press and saw at the movies. In other words, it took no more stress in these particular individuals than the simple declaration of war to bring out definite psychotic symptoms and break down their adaptation to life. At the present time we find individuals who have been making a good adjustment to civil life under demands which have not been too successful, but who have developed all kinds of anxieties and hysterical manifestations under the stress of mere anticipation of being drafted. A mere threat of war is enough to break down what appeared to be a good adjustment to the demands of civil life. Many individuals of this type will be weeded out; others will get into active service and will go to the camps. There they will be subjected to the stress and strain of making an adjustment to army life in an environment where discipline is strict, where they have to adjust to groups of individuals living intimately, dressing and undressing and looking after their personal needs under conditions that are embarrassing to the shy and diffident individual, and being subjected to teasing, razzing, or becoming the "goat" of the crowd. These individuals, able to go through the first stages, begin to break down when they are subjected to stresses and strains of army life. Then, after war is declared, and as we begin to embark and think in terms of getting overseas, another group of individuals begin to break down anywhere from the port of embarkation up to the front-line trenches. There were, however, individuals who went through all these phases with no evidences of failing to meet the responsibilities and obligations of army life, but who broke down on the way back to the safety zone—a sort

of delayed reaction. If the war had continued, there would undoubtedly be many more individuals hitherto intact mentally and physically who would have developed neuropsychiatric disorders.

Studies made of the constitutional background and the psychopathic taints do not show a great deal of difference in the hereditary background of those who broke down and those who were sufficiently stable to carry on. In general figures it can be said that we found 5 per cent of poor inheritance in the "shell-shocked" soldiers. compared with 2.5-3 per cent in those who were considered stable. There was a difference, but it was not marked. There was, however, a marked difference in this respect between the soldiers who broke down overseas under battle, under the stress and strain of the firing line, and those who broke down before they went overseas. The latter showed a marked constitutional neuropathic disposition, and their neuropathic background indicated that they were definitely unstable.

In my experience, "shell-shocked" soldiers seen en masse on the wards did not measure up in their personality make-up to the average sick soldier cared for in a general medical hospital. If you went into a hospital for "shell-shocked," you would recognize in a relatively short time that you are dealing with a group of individuals who, on the whole, are rather inadequate. It is interesting, too, to keep in mind the effects of different types of psychic trauma on different individuals. We find a great many men who went through the war, went "over the top," were right in the thick of battle, were subjected to all the strains of active warfare, showing no evidence of strain, who came back and developed a severe neurosis during the economic depression which followed demobilization. We found many men carrying on perfectly well as soldiers overseas, who would crack-up after hearing that their wives were sick or that the son died. Different individuals seem to be affected by different circumstances, stresses, and strains. One individual seems to be more susceptible to fear and others to grief. A threat to one's reputation, loss of self-esteem and prestige are factors that affect some individuals more than fear or grief.

During the last war about 2 per cent of those engaged in active service overseas developed "shell shock." One may say either that the 2 per cent represented the wholly unstable group, or that the other 98 per cent were unusually stable. I think it is logical to look upon the 98 per cent of soldiers who withstood the test of active service as representing the normal individuals, while the 2 per cent, for some reason or other, represented the unstable group.

It is of interest to point out that those individuals who represented the top 10 per cent in their psychological examinations as given in the camps were the individuals who, on the whole, made the best soldiers. They were better adjusted, and more frequently were selected as noncommissioned officers. In the lower 10 per cent were found the maladjusted group, the individuals who got into difficulties. We found these individuals more frequently in the guard houses and among those who went A.W.O.L.* They were the boys who were teased and harassed by their companions. It is among this type of men that the "shell shock" was most frequently found. I think this observation will be substantiated by the observations made in camp as time goes on.

*"Absent without official leave."

The neuropsychiatric casualties at the front varied in numbers and depended a good deal upon the severity and duration of the preliminary engagements and skirmishes which preceded big drives. At Chateau-Thierry, for example, about 20 per cent of the casualties were neuropsychiatric casualties, because the man had been subjected week in and week out to stressful experiences before they went into the actual battle.

It is recognized that a neurosis serves a purpose in the life of the individual. It has a protective mechanism. Trotter used the term "herd instinct" to denote such attitudes as loyalty, patriotism, honor, etc. He pictured this herd instinct and the instinct of self-preservation as the two forces which came in conflict and could only be dissolved by the neurosis. We tried to evaluate just what purpose the neurosis served in a soldier.

If one thinks in terms of the war neuroses as being a protective mechanism, we have to go back some years. The war neuroses were first recognized, I believe, by the Russians in 1905 and 1906, and provisions were made to care for these neurotic cases. That was the first time any recognition was given to the fact that war produced mental casualties and that these mental cases needed consideration on equal terms with other types of war casualties.

In the last war, the medical men, the military authorities, the public, and the press accepted the fact that "shell shock" was an illness not due to any malicious intent on the part of the soldier. Thus, the "shell shock" became by the same token a legitimate way out of a difficult situation. When war began, the herd instinct was a major driving factor for most of the men who enlisted or were drafted in the army. The feeling of self-esteem, the honor in which the individual wants to be held by his friends, by the community, by the military authorities, and by everyone else with whom he comes in contact, and the propaganda, speeches, the bands and parades, the uniform, the pride in being a soldier, the fact, too, that becoming a soldier oftentimes solved a good many conflicts of one's civilian life, such as worries and anxieties of an economic situation, or of a marital or professional difficulty, all those things contributed in building up this herd instinct. To join the army, to go to war, was the thing to do. It gave the individual a sense of importance and a sense of security. It enhanced his feeling of self-esteem. Nothing else mattered. The herd instinct as it related to the war was high, and the instinct of self-preservation was quiescent. One was not thinking in terms of life and death but rather in terms of satisfying certain emotional drives. However, as one became more actively engaged in military service, as one approached from the camps to the ports of embarkation, certain aspects of the glory and the glamour of the uniform were lost by doing kitchen police work and being subjected to an iron-bound discipline of the army. A good deal of this herd instinct began to fade away. The enthusiasm began to subside, and the instinct of self-preservation began to rise. As they neared the front line, the herd instinct became counterbalanced by the instinct of self-preservation. In those individuals who carried on, of course, the herd instinct was always higher, and the instinct of self-preservation remained submerged, while in those individuals in whom fear, anxiety, the emotional tension were of such a degree that the sustained threats

to their physical security could not be held in check by the herd instinct, severe emotional conflict resulted. The "shell shock" or the neurosis served the purpose of satisfying both drives, for it was recognized as a respectable way out of a difficult situation. It allowed the individual to protect his own self-esteem, because "shell shock" was looked upon as a legitimate cause for military incapacitation by the officers, by the doctors, and by brother soldiers, and at the same time it satisfied the instinct of self-preservation. It was a solution of an intolerable dilemma.

There were three ways of solving the conflict between the instinct of self-preservation and the herd instinct. One was to get killed, the second was to get wounded, and the third was a neurosis. We rarely saw a "shell-shocked" soldier, a neurotic soldier, who had a wound. The wounded individual didn't need a neurosis. He was out of the situation by virtue of his wound. Occasionally, the neurosis developed among wounded soldiers when the time came for their discharge, and they were confronted with the situation of having to go back into active combat. On the whole, one did not find neurotic soldiers among wounded.

Another interesting observation is that the neuroses were less frequent among the professional military men than among men who went into military service from civilian occupations. Thus, the English Army was, so far as the officers were concerned, made up to a large extent of professional soldiers. It was somewhat different in our Army where an officer might have been an insurance clerk or a lawyer or a business man, who went to one of the R.O.T. camps, and in the course of six or nine months became an officer. I do not believe I ever saw an hysterical paralysis in an English officer. I have seen a few neurasthenics, but, on the whole, the war neuroses were relatively few among English officers. They were trained for a life job, and consequently, were better adjusted to stresses and strains of war. Among the American officers, however, we often observed individuals suffering from a paralyzed leg, paralyzed arm, blindness, or some other hysterical symptoms. I remember, also, two or three high ranking medical officers coming to hospital with an hysterical paralysis, the sort of thing you would not believe compatible with a physician's knowledge of the nature of hysterical symptoms.

The neurasthenia and hysteria were the most common types of neuroses encountered during the last war. We saw relatively few cases of conversion hysterias with physical incapacities like paralyzed arm, leg, blindness, etc., in officers. We saw them frequently in the soldiers. A soldier with hysterical incapacity invariably represented an individual who met and solved the conflict between his herd instinct and his instinct of self-preservation at once, without putting much into it. The neurasthenic was, on the contrary, an individual in whom the internal elaboration of neurosis was more insidious. He went in and out of the front line trenches, he may have a good many symptoms such as insomnia, perhaps some battle dreams, feeling of exhaustion and fatigue, tremors and other symptoms, but he kept going in and out until he reached the point where he was no longer able to carry on. The hysterical individual met that situation more crudely. He was satisfied oftentimes with a group of symptoms that seemed so completely incompatible with his education, his back-

ground, his whole understanding of what that situation meant. The hysterical soldiers, as a whole, were more easily treated than the neurasthenics. They recovered from their symptoms more easily also, because they were eminently suggestible individuals, but they were also very much less likely to remain without relapses.

It was recognized early in the war that acute cases of war neuroses had to be dealt with promptly. It was found that cases with anxiety states, with hysteria, and with neurasthenic symptoms, which were sent back to the general hospitals along with the general medical and surgical cases were most difficult to treat effectively. Oftentimes they would be picked up with the general medical cases and simply remain for weeks on the wards of a general hospital, getting little or no psychiatric attention, their symptoms becoming fixed, and as a result of being in the wards with medical and surgical patients with organic symptoms, the whole picture became more elaborate and difficult to treat. That situation was bad also for the physically sick patients, as you know. For these reasons, receiving centers were set up within 5 to 10 kilometers from the front lines with psychiatrists on duty who saw all sick men as soon as they came back from the front. Thus, many of the soldiers who developed neurotic symptoms in action got no farther back than perhaps 5 to 8 kilometers from the front lines where they were treated. Many of these were cases of acute fear reactions plus exhaustion and fatigue. From 60 to 70 per cent of all such cases were returned from these centers back to the front line trenches within forty-eight hours. These centers did a useful work in segregating the cases. If all the neurotic cases had been indiscriminately evacuated to base hospitals, permitting a long period for the neurosis to become set before treatment could be started, undoubtedly many soldiers with milder neurotic states would never have returned to the front, and a large number of soldiers who well might have developed a chronic neurosis were thus salvaged for the service.

The amnesia cases represented a particularly interesting group, because these individuals attempted to solve their conflict between the herd drives and those of self-preservation by completely obliterating the memory of the intolerable situation. "Forgetting" allowed the man to carry on with duty, and one rarely saw the amnesic patient with any other neurotic symptoms. Sometimes the amnesia would be restricted to a rather short period in the individual's life, covering a space of time of perhaps a day or two preceding some trying event and extending beyond for two or three days. Other individuals would have a complete anterograde and retrograde amnesia lasting for weeks and months, forgetting completely all that happened before and after the event which produced the amnesia. There were also peculiar types of partial amnesia with confusion in which the individual would remember people and certain incidents but couldn't identify them with any particular situation.

The therapy of war neuroses varied with the different schools of thought. Some felt the approach to these problems was through analytical procedures and others felt that suggestion and hypnosis were more useful. It was important that patients got in and out of the hospitals as quickly as possible, and

one had to think in terms of methods that were practical. For that reason, a large number of the conversion hysterias who were undoubtedly precipitated by suggestion were treated by countersuggestion aided by faradic current ("suggestion armée" or "torpillage" method of the French). A patient in capacitated by an hysterical paralysis of the leg would be carried along at the hospital somewhat as follows: The doctor would take a history and endeavor to get on friendly relations, finding out all he could about the early history of the soldier's home life, interests, hopes, ambitions. attitude toward the war, resentments, attitude toward superiors, homesickness, fears, etc. Then a careful neurological examination would follow with any other examinations and laboratory tests that seemed advisable. Every psychiatrist had his own technique of treating his patients. One might start out as follows: "Now, John, you want to get well. You want to get over this trouble. You don't want to carry a bad leg around with you all your life, do you?" John would usually agree that he did want to get well. He would be taken off the ward to a treatment hut with a medical sergeant or corporal, and the psychiatrist would make another short speech, saying, "John, we are going to treat you and you are going to be absolutely cured when you go out of this place. That, I take it. is what you want?" Nothing short of that dogmatic assertion had any real value as a suggestive measure. John would say "Yes," a little less enthusiastically than he had before. There are a few psychological tricks, such as, "Lock the door, Sergeant." Then you tell John that the treatment may be very short or it may take some time, "It may take five or ten minutes, or it may take all day, John, but when you get out, you are going to be well; that is the important thing." "Now, John, this may be a rather painful procedure—does that bother you?" He would usually say "No," he just wanted to get so that he could walk. The hysterical patient was always very urgent in protesting against the illness and wanting to get over it. The electrical current would be applied and increased as found necessary, with continuous suggestion that function was to be restored. It is purely a matter of suggestion, and in a surprisingly short period, with the use of the current and help from patient, the muscles would contract; then the contractions would take place without the current. You show him, talk about it, demonstrate to him that there was function. Under the stimulation of the current the leg would jerk, and he would get cramps, some of them painful, and under the excitement he would find that he could move the leg, stand on it, walk. It was always desirable to spend a certain length of time with the patient after function had been restored, explaining the purpose of the paralysis, how it may come back under stress, and ways and means of preventing a recurrence of difficulty. After restoring function, the patient always was returned to his ward for the psychological effect on other patients. Went out in a wheel chair, returns under his own power! On the other hand, if a patient was not cured at once, he would not be returned to the same ward for obvious reasons. The neurasthenics and other types of neuroses did better under some modification of analysis, persuasions or re-education.

Sixty-five per cent of the cases of war neurosis were sent back to active duty, 30 per cent went to the service of supplies, and 5 per cent were found

unfit for military duty. The classifications used for the neuropsychiatric disorders were neurasthenia, psychasthenia, hypochondriasis, hysteria, anxiety neurosis, anticipation neurosis. Most of the anticipatory cases were those individuals who developed neurotic symptoms before they had engaged in any actual war activities. The individuals suffering from effort syndrome or neurocirculatory asthenia were common. They are being recognized at the present time by the examining boards and are considered as being unsuitable for service. There were many cases of exhaustion associated with a mild anxiety state.

I believe that the effects of the present war will not be particularly different from those seen in the last war. It is difficult to see how we can eliminate the unfit when the time of psychiatric examination is limited to six or eight minutes. In many of these cases the unfitness of the individual has to be determined by the evidence obtained from the individual's past history rather than on the basis of signs and symptoms the psychiatrist may discover during a short examination. Already examiners are finding men in the Army who have been in state hospitals or were released from state hospitals only a few weeks before their induction into the service. The attitude of some of the military authorities is still expressed thus: "You give us the men, and we will make an Army out of them." The fact, however, is that a man may be able to make a very satisfactory adjustment to civil life, and yet this is no guarantee that he will make a good soldier. The demands of the Army on the individual are quite different from those of civil life. Many a neurotic individual makes a fairly good adjustment to civil life, he may fit into the social scheme of things, he may be a pretty good father, husband, friend, and neighbor, and yet he is quite unsuited to meet the demands of military life. It is not possible to eliminate all the potential psychoneurotics. A certain number of them will be picked up before they get into service, others will break down when they get into camps, and still others on the way to battle lines. On the whole, it seems to me we are going to be confronted with the same kind of problems during the period of mobilization and active service as we had in the last war.

I spoke of a period of mobilization and of active service. I think the period of demobilization is a period quite as important in creating the neurosis as any other in the experience of the soldier. The methods utilized in demobilizing, and the various services that the government has set up for compensation treatment of war disabilities, for training in various professions, have created potent motives for neurotic manifestations in certain individuals. I believe that following the last war these procedures of demobilization created many neurotics. When you pay a man for being sick, penalize him for getting well, when you push him into highly specialized organized occupational schools, giving him a false idea that he is going to become a good engineer, good public accountant, while he is totally unsuited for that sort of thing, you create a great gulf between man's fanciful ambitions and the grim reality of his actual abilities to achieve these ambitions.

The war neuroses are not different from the neuroses we see in civil life. I think, however, that war neuroses are much more amenable to treatment than

the neuroses seen in civilian life. Created by suggestion, the war neuroses are readily helped by suggestion. There is a certain prestige of being an officer and in utilizing the therapy of suggestion. There is a greater prestige relationship between the soldier and the medical officer than there is between the patient and the doctor in civil life. In civil life, if the patient doesn't like one doctor, he can seek another. The soldier has no such rights. An important lesson we learned from the last war is that the nearer these neurotic manifestations can be treated to the place of their occurrence, the easier and quicker they are cured. If the neurotic individuals are treated at the receiving centers immediately behind the front lines and soon after their symptoms developed, a great many of them can be sent back within a short time. On the contrary, the most difficult cases to deal with were the cases evacuated from the front lines to general hospitals far in the rear. They just hung around and simulated symptoms seen by them in others, while their own neurotic manifestations would become chronic and fixed.

Question: Was there a difference in the incidence of "shell shock" and neurosis in the English and in the American troops?

Answer: I don't think there was any significant difference between the Americans and the British. We did, of course, make more careful examinations, poor as they were, before the induction into service than the British did. In England the men were thrown into service at first without much time for psychiatric examinations. They did have the same sort of sifting process in the British Army but not to the extent that the American Army had. The incidence of neuropsychiatric casualties was not particularly different.

Question: What immediate measures did you use for treatment of neurotics at the front lines?

Answer: Right at the front trenches these men were given rest. Reassurance was an important part of treatment. A good many of them had fear of being looked upon as cowards, and they were anxious to get back. Sedatives were used. It was a matter of bucking the morale of the discouraged soldier. Two out of three soldiers who came there from the trenches would be relieved after a night's sleep, rest, and reassurance that they were all right and not "yellow," and back they would go. They rarely were kept more than seventy-two hours.

Question: Did they go back to the same posts in the trenches or would they be transferred elsewhere?

Answer: About 65 per cent of the cases would go from the "triage" centers back to their own companies. The other third would be evacuated to base hospitals.

Question: Would a man be reassigned from the combat duty to kitchen or something like that?

Answer: This was done in some instances. They would get a Certificate of Disability marked one, two, or three, i.e., they were to go back to duty, or duty behind the lines, or they would be discharged. A number of those men were assigned to some type of service behind the front line.

Question: There is probably no other field than that of psychoneurosis in which the racial characteristics present themselves more distinctly. Is that your impression also, so far as the war neuroses are concerned? For example, the symptoms of conversion hysteria, such as hysterical paralysis, contractures, fits, blindness, seem to be more frequent among Latins than among Anglo-Saxons.

Answer: Yes. I think that is perfectly true in civil life as well as in military life. I suppose that is why method of "suggestion armée" or "torpillage" was originated by the French, for it is a method of treatment particularly suitable for conversion hysteria. We see relatively few cases of conversion hysteria in our large out-patient clinics. I doubt if there are seen more than a few cases a month in all the clinics of Boston of real conversion hysteria, while in the French clinics, I am told, they are seen much more frequently.

Concluding, I wish to repeat that the individual who develops a war neurosis, the malingerer, the fellow who tries to bluff his way out of the Army, the psychopath, the unstable individual, will not be a good soldier. It is far better to have on the soldiering job a man physically sick than a neurotic. The Germans don't recognize the war neurosis as sufficient reason to get out of service. They are using the neurotic in building roads and front-line trenches, under strict military discipline, subjecting them to all the dangers of front-line activity. We have no way of knowing how efficient such military measures really are in treating a group of inadequate individuals whether they be malingerers or neurotics. We do know, however, that these individuals do not make good soldiers. They certainly represent a menace to any organization, and neither the British nor the American Armies will probably resort to such measures.

"SHELL SHOCK" AND EFFECTS OF HIGH EXPLOSIVES*

D. Denny-Brown,† M.B., F.R.C.P.

THE term "shell shock" is rapidly becoming obsolete, though the clinical conditions it used to describe are by no means uncommon in the present war. Until I was asked to give this talk, I had not used the term for almost two years, following the findings of a British Commission which was set up in 1939, which recommended that the term would be dropped altogether. In the last World War millions of men in closely packed trenches were subjected to tremendous bombardments for months at a time. A mysterious kind of nervous disorganization occurred in those who were submitted to close shelling, and it was natural that this clinical syndrome should be thought to be due to the physical effects of explosion. It was difficult to deny some particular influence on the brain from the explosive force of the shell quite apart from direct injury to the brain from fragments. The nervous symptoms were often absent in those who had suffered severe direct brain injury. There began a search for some objective basis. Most of the patients suffering from shell shock did not come to autopsy. They were disabled for long periods of time, many of them with frank hysterical or neurotic symptoms, most of them labeled "chronic neurasthenia," some suffering from traumatic intellectual impairment, and some truly psychotic and requiring commitment. In the aftermath of the last World War thousands continued to attend special clinics and hospitals, receiving various degrees of pension for this disability, "shell shock." In the brains of the few who died without sign of external injury, pathologists reported finding in some no lesion of any kind and in others small petechial hemorrhages in the brain substance. It is now felt that such pathological changes as were reported were not necessarily present in those who survived and suffered from the clinical picture which was called "shell shock." Thus, in a case described by Mott, the man died from an explosion in a munitions factory but was known to have been exposed to carbon monoxide, which alone could account for petechial hemorrhages in the brain substance. Others may have suffered from fat embolism which also gives rise to such hemorrhages. At the time of the Spanish Civil War there came reports that bombing may cause death in a very mysterious way; tales of patients found dead after the bomb explosion who showed no sign of external injury and in whom at autopsy no cause of death was to be found. Again it was supposed that some mysterious effect of high explosives damaged the nervous system. One was naturally very skeptical of such reports, because in ordinary civil life dislocation of the cervical spine with transient compression of the spinal cord may cause death without more than a small softened segment. And so it is suspected that

*Lecture XIII, reprinted from *Collected Lectures*, the Metropolitan State Hospital, Waltham, Mass., 1942.

†Professor of Neurology, Harvard University, Major R.A.M.C., now demobilized for research investigations.

such dislocation may have caused these mysterious deaths in people who were killed by sudden explosive violence. Another rumor dating from the first World War was that if men were in a trench and a very high explosive shell fell near by, then in some way the ground was shaken and the people in the trench were concussed. It is difficult to find any adequate foundation for this, which is obviously related in some way to the problems of "shell shock" and unexplained death from bombing.

There are many points of resemblance between "shell shock" and "traumatic neurosis." As you know, many who have had a head injury in ordinary civilian life are particularly liable to nervous symptoms for a number of years afterwards. That condition has also led to differences of opinion. In its prolonged posttraumatic anxiety symptoms and variable association with actual cerebral damage, this syndrome is seen to be closely related to that of "shell shock."

I think some case histories will best give an idea of the nature of the problem. Most patients close to an explosion of bomb or shell suffer from direct wounds, such as foreign bodies in limbs, body, or skull, fractures of limbs, and injury to the chest. If one looks for what we call pure "shell shock," one must seek the patient who had no external injury from the explosion but who was unconscious. There are very few such patients, and their persisting symptoms are of emotional type. Many have had a definite but trivial injury, such as a cut scalp, and suffer later from the same symptoms.

Case 1.—An infantryman, aged 29 years, had survived several severe bombings without any symptoms at all. On May 30, 1940, a shell dropped close to him in a trench. He estimated that he was about twelve feet away. He remembers a loud explosion, and then he remembers nothing more. His next memory was three or four hours later, and about a quarter of a mile behind the line where this explosion occurred. He was shaking all over, sweating profusely, and, in his own words, "crying like a baby." From that time onward his memory is clear. He thus had suffered an immediate amnesia of three to four hours without retrograde amnesia. He managed to get to a hospital. He had no sign of external injury anywhere, no cut of the scalp and no bruise; but he had a very severe headache, and he was kept in bed for some days. He was very sick and nervous. He slept very badly for the first few nights. Whenever he heard aircraft go past, he was overcome by general trembling, and from time to time he would break down in tears. His thoughts continually were about another soldier for whom he had a particular liking, who had been killed the day before his own incident. He continued to have early morning headaches. He was extremely unhappy. His eyes felt heavy. He continued to sleep badly, and he was still the same about six weeks later when I saw him. He had good intelligence, having reached top grade in primary school at the age of 14. He had had two years in an engineering firm, then joined the regular army, served in Egypt, enjoyed it, and left the regular Army as a reservist and worked in a brewery. He had been married for several years, had two children, and his domestic life was perfectly happy. There was no history of previous breakdown nor of nervous symptoms or epilepsy, nor was there any family history of fits or breakdown. He had never had any illness that he could remember. On examination he had no abnormal physical signs of any kind. X-ray examinations of the skull showed no signs of damage. The cerebrospinal fluid was perfectly normal in all respects. After discussion of his troubles, and particularly his anxieties about his friend who had been killed, he settled down rapidly, and within a fortnight was free from headache, and was cheerful and slept well, and after a month was sent on sick leave. The last we heard of him he was back on duty without symptoms. What was the cause of this

amnesia? Had he been concussed or had he suffered simply an hysterical episode? I have no doubt that he was thrown into an acute emotional reaction by the circumstances of this explosion. His anxiety about his friend possibly predisposed him to this. Are we to call that "shell shock" or not? Is that a mysterious organic reaction of the brain, or is it purely an emotional phenomenon?

Case 2.—The next case was that of an infantry soldier who had been on guard duty when a bomb exploded some ten yards away from him. When he came to the hospital, he gave us a story that he had been unconscious for four days and had from that time onward suffered from severe headaches, from giddiness, whenever he ventured out of the hospital, and from inability to concentrate. He was at times depressed, and he did not sleep well. His personal history was a poor one. He had not attained beyond the third standard grade at school. He had tried all sorts of odd jobs until he had joined the Army, and he had a history of one brother who was committed. He had no history of previous breakdown. He showed no sign of damage to the nervous system or even to the scalp. X-ray examinations of his skull were perfectly normal. The only abnormality was an electroencephalogram which showed many slow waves of the kind found in psychopathic personality. Indeed, he was mildly psychopathic as well as backward. Mental status showed that his memory and judgment were particularly poor. Was this man concussed by the blast of the explosion, or was he suffering from an emotional nervous state like the first one? Eventually I interviewed another soldier who had been with him on guard at the time of the original bomb explosion. I told him I was rather interested to know about the "unconscious" condition of the patient during these supposed four days of unconsciousness, because we had no record from any hospital about this state. The friend said he had heard the patient shouting and ran to where he had been thrown into a heap of soft lime by the force of the explosion, and where he was shouting to be helped out. He was sufficiently conscious to be cursing the lime, knowing perfectly well what it was. It was then clear that this patient had not been really unconscious, and the loss of memory covered a period in which he had been extremely nervous and frightened of further bombings. That amnesia was not the amnesia of true concussion; it was amnesia of hysterical reaction.

These two patients are the only clear-cut examples of which I have kept a case record. But their story is enough to show what sort of reaction occurs in the patient who has not suffered external injury, but who is complaining of nervous symptoms following bombings. In strong contrast with these histories are two others characteristic also of close exposure to bomb and shell explosions but with penetration of the skull by fragments.

Case 3.—A ground engineer was standing on an airdrome near a plane when an aerial bomb exploded ten yards away from him. He stood clinging onto the aircraft, which had caught fire, but he could not speak. He does not remember any pain at the time. He had multiple injuries to his limbs, which were bleeding, and in the course of a few minutes he collapsed, but he did not lose consciousness. He remained conscious but unable to speak until after he was admitted to the hospital about one-half hour later. About six hours later he was semiconscious and almost completely aphasic with a right facial weakness, but no weakness in the limbs. He had extensor plantar responses on both sides and strong neck rigidity. He could understand parts of your conversation with him and simple requests, but he was quite unable to make any reply. The cerebrospinal fluid was blood-stained, under a pressure of 190 mm. After a few more hours he was able to give his name, and the next day he was able to name a match box. He was unable to write his name and he had no apraxia. The cerebrospinal fluid pressure had now increased to 340 mm., and the fluid was still blood-stained. X-ray examinations showed small metallic fragments had entered through a very small opening in the skull and had traversed the ventricles to lodge in the region of the basal ganglia on the left side. They had crossed the cranial cavity, including the body of the ventricles. The scalp wounds received surgical treatment, but

the intracerebral fragments were not disturbed. In three more days he was able to give his name and age; he was a little confused, said the year was 1933, and a little later said the month was June, 1940, which was right. He showed little perseverance in speech. In the course of ten days his speech came back. His right plantar response remained extensor for a few days. His cerebrospinal fluid became yellow and clear within a week after the injury, and he was feeling well. There was a slight trace of right facial weakness in expression but no other sign. He remained well at the end of three weeks, and other wounds in his limbs had healed well. He was allowed a gradual convalescence and eventually went back to duty feeling fit. A letter from him after he went back to duty at an airdrome revealed that at first he did not feel sure of himself, but he soon overcame this.

This patient had been very close to a large explosion, and his symptoms, all except some slight loss of confidence on first return to duty, had all come from contusions by small penetrating fragments which had entered the brain causing aphasia. Any amnesia was exceedingly brief.

CASE 4.—A sergeant in an artillery regiment was in the Metz area of the Maginot Line on the last day of April, 1940, when a shell burst a few yards behind him, throwing him forward. He did not recall the noise of the explosion, but remembered raising himself up from the ground. This was at night. He seemed to see a moving cloud, and for a second he felt unable to move the right arm, where he had sustained a flesh wound; he also found he had been wounded in the left leg. He noticed the right occipital region was beginning to bleed. He walked unaided to a post about twenty yards away. In the post he could see the light of a lamp and no more. His wounds were dressed, and he was sent to a field ambulance unit. He had no headache and his speech was normal. The next day the occipital wound was excised, and a gap in the skull in the occipital region was found with brain protruding through. No exploration of brain was made. He remembers seeing a nurse's cap to the right side of him at this stage. He was transferred to another hospital. He was apathetic, he did not sleep well, and his head wound was bulging but was not infected. He was found to have a complete left hemianopsia. He was receiving sulfapyridine, his temperature was normal, and he could now see faces. On May 22 he could see well to the right but not to the left, and his sight improved gradually. He was reading normally three weeks after the injury. He had no hallucinations. He had a headache for five or ten minutes a day at first, but after three weeks he had none, and he was well except for his hemianopsia. This patient had a large bone fragment in the right occipital lobe. There was here nothing of the condition called "shell shock" in spite of his close relationship to a shell explosion.

Many other histories could be given of severe damage to the brain from close explosion without inducing the phenomenon of "shell shock." In one striking case, a soldier recovered fully with bomb fragments still in his brain, and only after an interval of some two months, under the combined stress of domestic worry and further bombing, he began to complain of the headaches, giddiness, inability to concentrate, and insomnia characteristic of the condition. If we turn to cases of nervous reaction with doubtful head injury from causes other than explosion, we find also that the persisting nervous or emotional reaction bears no relation to the structural damage caused by the injury.

Case histories of patients suffering from "traumatic neurosis" following closed head injuries of other kinds were then given. The development of the posttraumatic syndrome of headache and emotional instability was considered to be related either to reduced *intellectual capacity due to the injury*, or to constitutional predisposition to neurosis. The symptoms in no way differ from those suffering from the effects of bombing. Returning to the condition called "shell shock," we may see in it an emotional reaction to the catastrophe of the

explosion. If actual brain injury has occurred, the syndrome may also appear, and then may be determined in part by the residual cerebral damage. It must not be forgotten that an emotional reaction can occur on a basis of real reduction of intellectual capacity. But the most characteristic case of "shell shock" has no history of direct damage to the brain and no evidence of reduction of intellectual capacity. The condition is essentially one of anxiety neurosis and is best labeled so. The very name "shell shock" predisposes either to self-pity or to self-glorification in the soldier and is a potent factor in perpetuating the condition. The greatest advance in the subject is the abolition of its old name.

The amnesias following bombing are usually patently of hysterical kind. The frequent absence of true concussion leads to investigation of the nature of the amnesia. Experiments were cited to show that true concussion is a traumatic paralysis of nervous tissue caused by acceleration. The change in motion of the head striking a heavy blunt object, or being struck by such an object, is very abrupt. Small penetrating fragments of bombs or shells do not impart such acceleration to the head as a whole and therefore cause little or no concussion. Similarly, the air-borne force of an explosion, though very brief, does not accelerate the head quite as rapidly as a solid mass does, and so does not concuss.

The violence of the displacement of the body and head by an explosion may cause concussion by flinging the patient against some solid object, but there is nothing mysterious or unusual in that. Secondary injuries of skull and spine are common, and fat embolism of the brain from broken bones sometimes occurs. The causes of death from bombing injuries to the nervous system are often complicated. Thus, people may be truly concussed by secondary injuries, and in any case are likely to suffer from the emotional disturbance that we call traumatic anxiety state, traumatic neurosis, or simply anxiety neurosis. Such a psychoneurosis persists usually only in persons with psychoneurotic constitutional liability. It is necessary to assess the nature of the original injury and the degree of intellectual impairment persisting if organic damage to the brain is to be determined accurately. It is naturally of great assistance to have a good record of the original injury. Physicians and surgeons who attend the patient at the time of injury usually lack time to make accurate notes, but any account of the patient's condition when he is first seen is of great assistance afterwards. Amytal is often of assistance in unraveling hysterical amnesias.

With regard to treatment, I might say that most cases of emotional disorder will make a good recovery with persuasion, discussion, and suggestion. In many, some abreaction is essential. In severe cases, usually with bad personal previous history, an extensive investigation of their childhood anxieties is necessary, and in this type of case it is very difficult to get them back to duty involving explosions and bombing again. From the military point of view, the patients who are likely to get back to duty usually do so after quite simple treatment, provided their trouble is understood, provided there is time to go into their story and get down to the nature of their anxieties. They do not react well to faradic or simple suggestion.

Question: What is considered to be the pathology of a concussion? I was taught that in concussion there was some shaking up of the neurons, perhaps some disturbance in the microscopic structure of cells reversible in nature, so that by the time they got to post mortem nothing could be found.

Answer: Most people like to picture some histological change in their minds when they think of concussion. But there is no known histological change. That does not mean that it does not exist, but rather we prefer to describe it as a disturbance in function. Concussion is traumatic paralysis. It is rapidly reversible. It is unlikely that any stain will show such a rapidly reversible change in nerve cells.

Question: Is the incidence of shell shock as great in this conflagration as it was in the last—1914-1918?

Answer: It is very difficult to get any statistical evidence. For one thing, it is listed under anxiety state or traumatic neurosis or brain injury (when such exists). Patients are scattered in numbers of hospitals so that few see many together at any one time, but I should say my impression is that it is just as common as it was in the last war. On the other hand, I am satisfied that the avoidance of the name of "shell shock" and the treatment of the condition as a neurosis has resulted in the reduction of the number of cases with persistent disability to a very small number.

Question: It seems to me that it would be quite difficult in your treatment to gauge how quickly the patients should go back to duty, because if it is a case of concussion, they are liable to need a very prolonged rest before they take up their duty, whereas if it is not a concussion but due to neurotic features, they would get back to duty rather quickly. Is not that a difficult point to decide?

Answer: Yes it is, because it is impossible to lay down a hard-and-fast rule. Each case has to be judged on its own merits. When the patient starts getting up and having headaches, it has to be decided then whether he is attempting too much after a real brain injury, or whether he is becoming nervous. The most important single index of severity of brain injury is the duration of the true amnesia. If retrograde amnesia occurred, the posttraumatic amnesia was a true concussion.

Question: Do you think it is possible to pick a soldier who is not going to have a traumatic neurosis?

Answer: Of all patients who are still suffering from traumatic neurosis more than six weeks after they are injured, the number who have a bad family history or previous history is very high. The proportion with abnormal electroencephalograms is also very high, but they are not always the same group. Some have normal encephalogram and poor family history, and vice versa. Some have neither abnormality. It is unknown how many with equally bad histories fail to become casualties in spite of abnormal stress. That would be an important subject for study.

ALCOHOLISM IN MILITARY SERVICE

MAJOR MERRILL MOORE, M.C., U. S. ARMY

ALCOHOLISM and drug addiction among men in military service are usually concealed and thus are subjects one can approach only indirectly. A direct approach to them is doubly difficult, because it requires consideration of the whole personality of the men in service. Two contrasting extremes come to my mind when I think of the alcohol addict or the drug addict in military service. First is the picture one sees when the band begins to play and the regiment is out on dress parade. No one would think to see the stalwarts marching past the grandstand that there could be one drunkard in the entire outfit. The other picture is the one that the psychiatrist sees years later in the hospitals of the Veterans' Administration. As you know, many patients are admitted there on account of alcoholism or drug addiction. Somewhere between the parade ground and the Veterans' Hospital, alcoholism and drug addiction become a problem.

A considerable number of the patients admitted to Veterans' Hospitals are ex-service men whose illness has been diagnosed *alcoholic psychosis*. A smaller number of men are given either the diagnosis *psychosis due to drugs and other exogenous toxins* or *without psychosis, drug addiction*. The early stages of these conditions may not be obvious when men are in camp or on maneuvers, nor are they a prominent feature among the neuropsychiatric conditions encountered in actual combat. But, when the war is over and men are depending on their service record for hospitalization, a substantial number of cases of alcoholism or drug addiction will crop up expecting care for the rest of their lives. How did these men get into the service, assuming that they brought their problems with them, or how did their problems develop in service? How is it legally possible for them to claim service conditioning or service precipitation of their difficulties that arose afterwards?

First, one may consider the men who are already addicted to the excessive use of alcohol or drugs *before* they enter the Army. In the first World War men were taken into military service very rapidly. If they had a neuropsychiatric examination, it was often rather sketchy. Some might say that is still true. But neuropsychiatric entrance examinations are more complete today than they were twenty-five years ago, although sometimes a neuropsychiatrist still has only a very few hours in which to examine twenty or thirty applicants for military service. In doing this work, anyone can make a mistake, and when one is in a hurry, it is easier than usual. To prevent this, the time allowed for the examination of each case should be longer, and the form of the examination should be amplified.

Reprinted from The Military Surgeon 91: 29 (July), 1942.

Even though the examination may comply with the strict limits of the military forms that are filled out, it is still possible for a good many alcoholics to get into military service. There is no exact way of preventing this at the present time. Theoretically, the alcoholic is not good material, but occasionally one makes an adequate soldier. But too many alcoholics are not good for the Army, the General Grant tradition to the contrary notwithstanding. I wish someone would decide whether or not the Army is good for the alcoholic. It may be possible that some of the factors which have precipitated alcoholism in an individual case may be alleviated by military life. On the other hand, military routine may aggravate the alcoholic tendencies of some individuals. The Army, of course, is primarily a functioning defensive or offensive unit, and hence can concern itself only secondarily (if at all) with the therapeutic advantages of Army life for the individual. But, since the Army is made up of groups of individuals, their welfare in Army life becomes an essential part of the mechanics of the Army. The benefits a man gets from Army life depend on his own personality, the nature of his work, the way he is handled by his superior officers, and the vicissitudes of military life.

Today when men are being examined for the Army, there is a careful checking of outside sources in an effort to obtain some information about the man's personality and habits. The problem still remains, "How can the alcoholics be excluded from service?" In some cases a careful neurological or psychiatric examination gives little evidence of latent alcoholism, because if the alcoholic individual eats and exercises adequately, he may not show the physical effects of heavy drinking. Even if the examining officers communicate with the parents, the teacher, the minister, or the postmaster, a prejudiced opinion may be returned in favor of the man, especially if the family wants to get rid of him, or if he wants very much to get in the Army and they feel that enlisting is the only way out or is the best way to save his face in the community. These attitudes aggravate the situation and work to the detriment of the government. Many people tend to feel that if a man does not fit in anywhere else, he can enlist in the Marine Corps or go into the Army or the Navy, and that then he will be Uncle Sam's problem. Certainly he will then no longer be the problem of his own family or the out-patient clinic or the social service department of a hospital. People have come to me as a private physician to ask me to give favorable recommendations to selective service boards concerning ne'er-do-wells, latent or patent alcoholics, and various types of psychopaths who, in all probability, would adjust themselves no better in the Army than they have done in civilian life. I am sure that many other physicians in the United States have had similar requests. The public must learn that the qualifications for success in the Army are essentially the same as those in any other profession. Stable and mature persons are needed. It may be a fact that during the Napoleonic wars just anybody was good enough for the Army and was welcomed there, the fellow who got a girl in trouble and had to leave town, the poacher avoiding jail, and the criminal who did not dare show up again at home. Those men made a motley gang that was good enough for Boney, but that is no longer true today. Our Army is reorganized. It is streamlined. It is scientific, and it is psychological. *It wants and it must have the best.* A military life is not good shotgun

therapy, and the Army, Navy, and Marines do not want psychopaths, alcoholics, and neurotics, or persons with behavior difficulties. It is going to take some time before these points can be made clear to the public, but if that can be done, and if the present standards of admission can be rigidly enforced, twenty years from now there may be fewer derelicts suffering from the end results of chronic alcoholism and drug addiction in the Veterans' Hospitals of 1962.

When I speak of alcoholism in the service, I do not mean the kind of drinking seen when the American Legion is in town. That is something different. I would distinguish that as being a healthy kind of "blowing off steam," something that does not occur very often. In fact, it occurs only during the Legion Conventions. I realize that when the Legionnaires meet annually their emotions are very strongly stirred by convening and the recollection of old times. Generally speaking, the Legionnaires are not the type one would define as alcoholic.

The alcoholic has been variously described as a man who cannot get along with liquor and who cannot get along without it, or, he is a man who lets liquor run him rather than controlling his liquor. The alcoholic is a person in whom alcohol acts as a poison; he does not react to alcohol (physiologically) as a normal man reacts. He is a person who drinks to a degree that interferes with his work and home life. A final definition of an alcoholic is one who cannot stop drinking. These are all practical definitions; they are not scientific, but a jury would know what is meant by them and would understand the thread of common meaning that connects them all. We must use such definitions as these until more basically scientific facts are discovered. If an underlying basis for a definition can be found, it will probably contain some of these central ideas.

The problem of alcoholic addiction in military life is a good deal like the problem of alcoholism in business. If you ask a business man if alcoholism is a problem in his firm, he will say, "Certainly not. As soon as a man starts to drink, we fire him." But, if you say, "How many men have you fired during the last ten years for alcoholism?" he may be able to locate in his records a fairly large number.

In general, when alcoholism occurs in the Army, the drunk gets put into the guardhouse and is punished or discharged. The handling of him depends on the way the men live and on his rank, as well as upon other personal factors. If an officer becomes alcoholic, usually his friends take care of him, or he may be put at a post where he is not too important. Or he may not be advanced, or he may be retired prematurely. This occurs through the internal mechanics of military administration. In some instances, however, when alcoholism is acute, the individual is put into a hospital for observation and treatment. Alcoholism is accepted as one of the drawbacks inevitable in an imperfect world. However, some consider that excessive drinking in the ranks is an index of poor material or low morale, and that the best possible way to prevent poor material from getting into the service is by rigid entrance examinations and by scrupulous following up of the qualifications for admission to service that are already in operation. This applies to officers as well as to enlisted men.

Excessive drinking among enlisted men creates several difficult problems. These problems are not simply administrative or merely medical, they are basically psychiatric in their nature. Recently, I heard an officer discussing this

matter. He said, "Yes, heavy drinking among soldiers is a problem, particularly when you have large numbers of men who are not actively occupied most of the time. Under certain unfavorable conditions, it can become serious, but a lot can be done in a constructive way to control it. Where there are few troops it is usually not much of a problem. In general, it can best be handled in the following manner: First of all, military police are put on and the penalties for drunkenness are increased. Second, arrangements are made to give the enlisted men the use of facilities for recreation and diversion, such as swimming pools, tennis courts, bowling alleys, etc., through clubs and social groups; and dances, parties, and picnics are planned. We found that the more we saw to it that the fellows had a fairly good time, the less difficulty they had with the sort of desperate and destructive drinking that military men sometimes go in for." He added, "It ended with our enlisted personnel having better opportunities for recreation than the officers had, but the officers didn't object, because they thought the results were worth it. On the whole, it was a very good solution. Of course, it would be different under conditions of actual warfare."

Other officers who are familiar with disciplinary and recreational problems in military service corroborate this point of view.

In general, the men who cannot adjust themselves in military life and who succumb to excessive drinking are probably the same kind of inadequate individuals for whom some assistance and supervision have to be devised in civil life. And often the type of help needed by inadequate and alcoholic individuals in their efforts toward emotional and social adjustments are more than the ordinary civilian or military community alone can afford.

In the first World War these problems were beginning to be recognized. Now recognition is more widespread, and more of an attempt is being made to deal with them today than was made in 1917. The first emphasis is now being put on preventive measures. Yet, a satisfactory approach to these issues remains a partially unsolved problem in military life. When the first World War began, in April, 1917, there was no question about where things were heading. There had to be an all-out preparation for action, and the nation fell into a frenzy of the most patriotic and strenuous military effort this country had ever known. After a relatively short period of training, men were shipped immediately abroad and action was quickly forthcoming. All was excitement and activity. The problem of undrained emotional energy among the troops was not so severe as it has been until the declaration of the present war. Now we are really in a shooting war with a large and growing Army. The general staff is so busy equipping and training men at this moment that it cannot be expected to look after the individual emotional problems of service men. At the moment, this is a civil problem and has to be handled by communities, not only through the U.S.O., but through every community organization. Great progress has been made in the last six months along these lines. Until war has been actually declared, in some communities men in uniform are discounted socially. After the declaration of war, however, their stock goes up as war tension rises and the public begins to appreciate soldiers and sailors. Civilians in any community, with relatively slight effort, can do a great deal to turn the interest

of enlisted men *away* from the inadequate and destructive types of amusement and drinking that men in uniform sometimes fall into when they are on leave or liberty. Until such effort is made by civilians and community organizations to provide normal entertainment, excessive social drinking is apt to continue. The alcohol problem in military service is essentially an emotional or personality problem, just as it is in the civilian life, though it is also directly related to the release of unspent energy, in recreations and diversions carried out by individuals or groups in sports, games, dancing, and all social activities. Although social measures do not get at the roots of the alcohol problem, I believe that 95 per cent of the men who drink too much while on leave, for example, would accept nonalcoholic entertainment just as easily. For that matter, alcoholic entertainment (in moderation) in a wholesome setting would be much better for them than drinking alone. Not alcohol, but the intemperate use of alcohol, is the problem in the army as well as in civilian life. People have asked whether or not beer should be sold near military posts. I, for one, think emphatically that beer should be sold and that drinking in moderation should be encouraged. Recreational facilities of all sorts are essential to men undergoing the strain of preparation for military life. Of course, someone will point out that this brings up the sex problem, but the United States Public Health Service is learning how to handle this quite adequately. In spite of progress, we shall always have with us the sporadic critic or the fanatic who will complain if a soldier off duty has a ten-cent beer or puts a nickel in a juke box.

One of the most important aspects of Army life is the relationship between the officers and men. I believe that one of the most effective approaches which a professional man can make to any situation is the psychotherapeutic or melioristic approach. This can be used whether one is a doctor, a lawyer, or an officer. This approach aims at establishing confidence between individuals and encourages the best possible outcome of the situation. This point of view is well-known to physicians in their work with neurotic and psychotic individuals. It has found a concrete expression in the mental hygiene movement, which consolidates the psychological findings of psychiatrists, physicians, social workers, and lawyers. This information is now available in a form that can be applied in the home, the school, and the community. There is no reason why some of the basic principles of mental hygiene should not also be applied in military life. Men learn a great deal from military service that they could not learn at home or in school or in the community where they have lived. A military program can and should include constructive outlets for the energy of the soldiers to balance the necessarily destructive aims of warfare. There is no reason why along with their other duties officers should not understand and use the principles of mental hygiene in their work. Essentially that is exactly what a good officer does. If a soldier looks back through his military experience, he will remember some officers who were outstandingly capable, constructive, realistic, and fair, and were a good influence generally on the men under them. In military life, as in civilian life, the influence of the superior personality is a very powerful force in molding morale and directing policy.

The problem of draining emotional energy for many soldiers is *not* completely taken care of by physical work and military drill. There are a great

many men who drill all day, fall out, turn in, and that is all there is to it. But there are also in service men who have a superabundance of energy. Work does not exhaust them. Furthermore, there is a mischievous type, who seems compelled to go out and have some adventure once in a while, to get into trouble or get drunk, or do something exciting. Even he is not basically the alcoholic type. The soldier who occasionally gets drunk and gets over it is not the one I mean when I speak of the alcoholic.

I believe the alcoholic is distinctly a sick person, a man with a sick or abnormal personality. His personality is far more unstable and more immature than the average. He stands out as distinctly different from his fellows. He may be the kind of maladjusted service man who is chronically complaining, who tends to blame others, who "can't take it," and he may show what the psychiatrist calls "paranoid tendencies." There are a few crazy people in the Army who get along perfectly well. They fight and go through campaigns; occasionally one may become a hero. But there is also the kind of person who never gets along anywhere very well. We have to fall back on that overworked word *psychopath* to describe him. The alcoholic and the drug addict are recruited from the ranks of the psychopaths. We have said that alcoholism is an illness; it may also be considered the symptom of an illness. By that I mean that alcoholism is a disturbance, a smoke screen, covering an underlying personality disturbance. To call it a disease is merely a simple way to acquaint people with the fact that we realize the alcoholic is a sick person.

If you study an individual alcoholic, it will be apparent his symptoms go back to his early life. I should like to tell you about one patient with whose case I am familiar. He is a man who has been committed now for about twelve years to a Veterans' Hospital. I am using his case as an example, because it illustrates several important points. The patient is now 49 years old. First his illness was diagnosed *alcoholic psychosis*, but after he had been in the hospital for a few years the diagnosis was changed to *dementia praecox, paranoid type*. He was born in New England and was an only child, whose father died when he was four. He graduated from college with high marks and was engaged once, but his fiancée jilted him. He served abroad with the Army of Occupation. He always drank a great deal and became drunk frequently, but he managed to get by because his friends covered up for him very well. He was known to have drunk to excess on several occasions before he was discharged. Once he was called before a neuropsychiatric examining board (shortly before his discharge), at which time a psychiatrist saw him and made a note in his record that the patient had a psychopathic personality with paranoid tendencies. After discharge from the Army he went back to work but did not get along very well. He began to drink excessively and got into several brawls. About 1925 he began to develop the idea that he had invented an electric machine that would do anything. Then he began to feel that people were trying to steal it and that he was being persecuted. Following this, he was committed to a private hospital for a period of study. At this time, the clinical picture was complicated by his drinking and by neuritis and other symptoms due to drugs he had been taking. This cleared up and he was released. Later he got into the same kind of trouble

again, and this time was committed to the Veterans' Hospital. When I spoke with his mother about him, she said that he was very sensitive as a little boy. He was overidealistic, perfectionistic, and he did not get along well with children his own age. He did get along very well with older people. She said he was always timid and that he was afraid of men, and he was also very reserved with girls. She could not get him to go to dancing school. He had a good deal of trouble with bed-wetting until he was 8 years old, and after that masturbation was a severe problem. Once she took him to see a child psychologist on that account. When he went to college, the school authorities reported that he was one of the men who was always drunk at any dance or social gathering. He would often get so drunk before a party that he would not even get to it. He would occasionally take a girl to a dance, where he would get drunk or start a fight, and somebody else would have to take the girl home. He was seclusive and not very popular socially. Out of a class of 100 men, he had only one close friend, who also drank heavily. His military service record was not remarkable. After the war he developed a frank psychosis.

I have taken as an illustration a man who started out with alcoholism and developed a definite psychosis. Looking back, I do not think anyone at the beginning could have said that this man was normal or that he was well. He was a problem child, a difficult adolescent with many neurotic traits, and probably had a deep-seated neurosis, which slowly developed, with social deterioration, finally resulting in the complete breakdown of the individual. Whether alcohol was the cause of the breakdown we do not know, but it was an important precipitating factor. Alcoholism combined with paranoia is usually a precursor of permanent mental illness, as turned out to be the case in this instance. This man has not responded to any of the efforts made to treat him. He turns his back on anyone who comes near him. He never speaks but just closes his mouth and sits. Occasionally he is violent and combative.

The results of attempts to treat the chronic alcoholic addict have not been very encouraging. The different forms of shock treatment have been tried without much effect. It is very difficult to say just what the alcoholic is or how he can best be approached for treatment. Usually the alcoholic individual may be classified with the psychopathic or the borderline group, though in some instances excessive drinking is a symptom of an actual psychosis. Many alcoholics for a long time appear to be neurotic individuals going through severe conflicts. They drink to escape their conflicts and the resultant nervous tension.

When a man drinks, the physiological and pharmacological effects of alcohol are very definite. First, alcohol enters the blood stream very rapidly through the stomach wall. Some of the alcohol is stored in the liver. It is believed that this fact may partially explain why some people can hold their liquor and others cannot. Tolerance may be an expression of liver function. For many years, Dr. Timothy Leary, at the Boston City Hospital, has studied cirrhosis of the liver in cases of chronic alcoholism, where it is a very common finding. He reports that the subject needs further study. One confusing point is that cirrhosis of the liver is sometimes found in persons who have never touched alcohol.

Next, alcohol passes into the spinal fluid by which it comes directly in contact with the nerve cells. Once it was believed that a man who had a certain

amount of alcohol in his blood was drunk, and if he had more, he was more drunk. This, of course, is true within general limits, but more careful study of alcoholic individuals has recently shown that one can take a drink and begin to get "tight" at a certain blood alcohol level. Then, when the blood alcohol level, after further drinking, rises, instead of getting more drunk, the individual seems to adjust himself to it mentally and may appear more sober. These observations need to be explained. Learning phenomena is not sufficient; there may be something more, something physiological to this reaction. These findings may limit the value that up to now has been found in "drunk meters" and alcohol breath tests, but this should not entirely destroy their use. Legally, blood and breath alcohol tests are still valid, subject to interpretations, and they are far better than nothing.

Different tissues, such as muscle and brain, apparently oxidize alcohol at slightly different rates. For example, a laborer may get over a drunk quicker than a brain worker, because he may burn it up in physical exercise out-of-doors. Furthermore, the oxidation rate of alcohol in the body may be somewhat altered by certain drugs, for example, dinitrophenol derivatives, and possibly benzedrine, though the evidence is not final. It is possible that some day a drug may be found that will alter the oxidation rate of alcohol in the human body in a way that may be therapeutically useful, though at present the available drugs are too toxic for therapeutic use. The factors that influence the lowering of the blood and spinal fluid alcohol content are all-important physiologically and need to be studied much more intensively. Generally speaking, the blood alcohol level in an individual is the result of two factors, the amount of alcohol taken in and the body weight, since alcohol is dissolved in all body tissues about equally. For example, if you take two men, one who weighs 250 lbs. and another who weighs 125 lbs., and give them the same amount of alcohol, the man who weighs 125 lbs. will have a blood alcohol level about twice as high as that of the man who weighs 250 lbs.

The next point to mention, when speaking about the effects of alcohol, is this: There is no evidence that alcohol increases human efficiency, except possibly momentarily. As the blood alcohol level rises, efficiency of the nervous system and performance gradually are reduced. From elation, drinking can lead to coma. Although we speak of getting "tight," we really get "loose" when we drink, because the basic action of alcohol as a narcotic drug is to depress the higher centers and release the inhibitions. There is a vast amount of misinformation about that one point. Forty or fifty years ago, physicians were taught that alcohol was a stimulant, and it is often listed among stimulants in old textbooks. Actually, alcohol acts as a depressant in its effect on the nervous system; the only stimulation alcohol affords is to the taste buds of the tongue and the mucous membrane of the throat.

From the point of view of military efficiency, this is significant. Many years ago alcohol had a very important part in the military diet. It has been served as grog in the Navy. Rum rations were given to Continental soldiers. Sam Houston is said to have fought most of his Mexican campaign on rum, and at the Battle of New Orleans it is said that Andrew Jackson was so weak (after a

strenuous march) he had to be helped up on his horse at first, and that before he could lead his men, his attendants gave him a mug of whiskey to drink. But the best information about the modern Army is that alcoohl plays no official role in warfare and is excluded from actual maneuvers. It has been reported, for instance, that the Nazi troops in active campaigning are not allowed any alcoholic liquor in any form, and that the picked troops who invaded France in May of 1940 were, for the most part, men who had been on a military training regime more strict than that of any football teams in our better universities. If this is true, it may be very significant. It may mean for all armies now that under serious military conditions the amount of drinking that is permitted may be greatly restricted or practically nil.

In summary, however, man's nature is not apt to be basically changed, even by warfare. In whatever form alcohol may be provided or available as a beverage, and whatever its effect may be, considered from the realistic point of view, it is present and is accepted as a part of life, along with food and drink, and the fact remains that for the great majority of the civil and the military population, alcohol does not constitute a problem of serious importance. The individual with alcoholic tendencies is the exception rather than the rule. What is important, however, is that drinking in moderation be encouraged or emphasized. Personal repressive and restrictive measures are basically of little use, since they fail to get at the roots of the problem. It is also important that reasonable and wholesome facilities for relaxation and recreation be provided men in military life. And, what is most important, that further effort be made to discover and understand the type of individual who is inadequate, who tends to drink to excess in the escapist, addictive, or destructive patterns, and to keep him out of the armed forces of the nation. He will still exist, of course, and will still remain a problem for the community to deal with, but his future is not as hopeless as it once was now that physicians are more clearly realizing that the alcoholic is a sick person and that he was sick *before* he began to drink, and that he is sick between bouts when he is *not drinking*. His illness can be further defined as a deep-seated disturbance in his personality, a long-standing difficulty, a maladjustment in his emotional or in his social life, sometimes the expression of an inner problem, or sometimes the result of an external situation in his life. In this sense, alcoholism can be regarded as a symptom, or as a smoke screen, for that is what it always turns out to be, when the individual is studied and an effort is made to treat him. About this basic disturbance of which alcoholism is the symptom, physicians know as little today as was known about diabetes forty years ago. By analogy, it is as important to keep out alcoholics of the armed forces, generally speaking, as it is to keep out diabetics. That is what physicians and neuropsychiatrists at the present time are trying to do. To some extent they are successful at this task, and to the extent that they are, there will be just that many *less* alcoholic derelicts and drug addicts to be cared for in government hospitals in the future. The men who are excluded, however, will probably remain in the community and will probably continue on a destructive or disintegrative path, as disturbed individuals without treatment usually do, but even there they are not being ignored. An effort is now being made in some

communities to study and treat these individuals with the aim of preventing alcoholism. This effort is based on the idea that they are sick, and that their sickness is accessible by three well-known avenues: chemical, psychological, and social. It is believed by those who are carrying on this work that the methods of science and a modern progressive and humanistic approach will not fail in time to produce worth-while results in terms of more satisfaction and usefulness for the alcoholic, and the reduction of unnecessary psychic suffering on the part of the alcoholic individual and those who are dependent on him.

AN ACUTE RESPIRATORY INFECTION RESEMBLING SO-CALLED ACUTE PNEUMONITIS*

A Report of 40 Cases

Lieut. Com. LeRoy B. Duggan, M.C., U.S.N.R., and
Lieut. William L. Powers, M.C., U.S.N.R.

During the months of June, July, and August of 1941, the department of medicine at the United States Naval Hospital, Corpus Christi, Tex., observed an acute infectious disease of the respiratory tract with unusual pathological changes in the lung.

There were 112 admissions with acute respiratory infections during this period, and the present study is based on 40 cases with the above unusual lung findings. These patients were young adult members of the United States naval military service and were between the ages of 17 and 26 years.

Cases which apparently simulate these have been reported during the past several years in the United States and foreign countries, e.g., England, France, Hawaii, and Spain.

The disease differs from the familiar forms of lobar pneumonia caused by pneumococci and from the usual types of bronchopneumonia caused by hemolytic streptococci, staphylococci, and other organisms. In the majority of the cases reported the course of the infection is characterized by an insidious onset with minimal respiratory and mild constitutional symptoms. At the onset there are no definite abnormal physical signs and the leucocyte count is normal or only slightly elevated. Within 48 hours a roentgenogram of the chest shows a small area of pulmonary consolidation usually located at the base of one lung.

This disease has been described by a variety of names, e.g., as acute influenzal pneumonitis,[3] acute pneumonitis,[1] atypical pneumonia,[5] acute interstitial pneumonitis, atypical bronchopneumonia,[11] peribronchial pneumonitis,[9] and adolescent pneumonia.[6]

In 1934 Gallagher reported a series of 16 cases with what he termed adolescent pneumonia and which had many characteristics very similar to this clinical picture. Bowen, however, was apparently the first to suggest that this infection might be a new disease entity when, in 1935, he described an outbreak

*Reprinted from the U. S. Nav. Med. Bull. 40: 651 (July), 1942.

occurring among Army troops stationed in Hawaii. In 1936 Allen, at Fort Sam Houston, Tex., reported a series of 50 cases with acute pneumonitis. In 1937 Scadding, in England, reported a group which he called disseminated focal pneumonia. In 1938, 1939, and 1940, sporadic cases and minor epidemics were reported in the United States and England. These outbreaks were widely scattered in this country, with isolated cases and minor epidemics being reported in Pennsylvania, Oregon, Texas, New York, Minnesota, Ohio, Delaware, Maryland, California, and Massachusetts. In 1941 small epidemics were reported from widely separated points in Texas and Oklahoma.

The majority of these reports describe cases very similar to those we have observed in our series. However, those described independently by Cass in 1936 and Reimann in 1938 had more marked constitutional symptoms than those we have seen. Also the series reported by Reimann and Haven in 1939 differed in that those cases associated with abnormal pulmonary changes had severe constitutional symptoms and those having mild symptoms similar to our cases had inflammatory changes in the upper respiratory tract. Symptoms due primarily to inflammation in the upper respiratory tract were not an important or frequent finding in our cases or in the majority of those reported by other workers.

At the present time there is considerable speculation as to whether these cases represent a separate disease entity caused by some type of filtrable virus or are simply atypical bronchopneumonias caused by one of the usual types of bacterial organisms. Since these cases occur largely in previously healthy young adults, Lyght and Cole have questioned not only that this infection is typical for this age group and type of individual, but also that it is a new or separate disease entity. They call attention to the fact that the concept of pneumonia, as a disease entity, can no longer be restricted to a well-defined clinical picture because both the nature and severity of the infection are subject to too many variables. It was their opinion that the concept of pneumonia should be broadened to include the type of case under discussion.

Although the etiology of this disease is still in doubt, the prevailing opinion seems to be that it is caused by some type of filtrable virus. There is some evidence to support this view as shown by the following, e.g.:

1. The contagiousness of the disease.
2. The inability to demonstrate reactions associated with any known pathogenic bacteria either by culture of the sputum and blood or by other laboratory procedures.
3. Failure of the infection to respond favorably to sulfonamide chemotherapy.
4. Work of Horsfall and Weir and Stokes and Francis with recovery of a virus and production of pulmonary consolidation in animals.

Stokes and Francis obtained an unusual virus from the nasopharyngeal washings of one of Reimann's patients and from the blood of another. It was virulent for mice and caused pneumonia and encephalitis about 2 weeks after inoculation. However, they were unable to do serological tests because of the loss of the agent and, therefore, were unable to establish a causative relationship.

Although Horsfall and Weir were not able to infect a variety of animals by intranasal inoculation with throat washings from a number of clinically typical cases, they were successful in transmitting the infection to the wild mongoose. They succeeded in recovering a virus which on inoculation pro-

duced pulmonary consolidation in the animal. Healthy mongooses placed in contact with infected mongooses developed pulmonary consolidation. The virus was neutralized by the serum of mongooses convalescent from the infection but was not neutralized by normal mongoose serum. Serum of human beings convalescent from acute pneumonitis also neutralized the virus, but serum obtained from the same persons during the acute phase of the disease failed to do so. The virus was filtrable through Berkefeld V. and N. Candles, was not inactivated by glycerin or freezing and drying in vacuum, and was propagated for at least 30 serial passages on the chorio-allantoic membrane of the developing chick embryo. It was the opinion of the authors that virus is the cause of so-called acute pneumonitis in human beings.

Enders, Sullivan, Hammon, and Meakins of the Department of Bacteriology, Harvard Medical School, attempted to demonstrate an etiologic agent by the inoculation of mice, rabbits, guinea pigs, ferrets and macao mulatta with blood, sputum, and nasopharyngeal washings from 11 patients in Murray's series of cases, but were unable to produce any recognizable pathologic changes in any of the animals.

The pathology of this disease is limited to the findings in one fatal case which occurred in Kneeland and Smetana's series at the Presbyterian Hospital, New York City. The microscopic features of the pneumonia were characterized by a mononuclear cell exudate in the pulmonary alveoli and an acute pulmonary vasculitis. However, none of our cases are comparable to their group of severe infections in which the death occurred, and therefore, any conclusions drawn regarding similar pathologic changes in our series of cases are likely not to be accurate.

Most of the reports of this disease have been made as a result of the study of outbreaks occurring among young adults living either in colleges or quartered with military troop concentrations. In these two groups a substantial number of cases have occurred over relatively short periods of time, and therefore, the disease has been thought to be readily transmissible. Although our report represents a similar situation, we were unable to find that the disease was any more prevalent among the men living in barracks with possible contacts than in those who were unexposed.

The disease occurred in about the same ratio in aviation cadets, was of about the same severity, and ran a similar clinical course. Enlisted men living at outlying auxiliary fields acquired the infection, but these cases were rare. Although we were unable to trace exposure or contacts of sufficient number to determine the definite degree of contagiousness, it was our opinion that it was probably transmitted from one person to another by casual contact.

There was no evidence to indicate that lack of physical fitness, previous upper respiratory infections, abnormal exposure, physical fatigue and climatic conditions were predisposing factors.

CLINICAL DATA

The average typical case began as a simple acute infection with minimal respiratory symptoms. Usually for a day or two preceding admission, the patient had not felt well. Following this, he developed a moderately severe

frontal headache, backache, pains in legs, weakness, unproductive cough, and fever (100°-102°). In the large majority of instances, these patients were considered only moderately ill.

On admission, physical signs were minimal or entirely absent. Usually there was slight redness and injection of the pharynx, and an elevated temperature. The pulse was either normal, or the pulse-temperature ratio showed a relative bradycardia. The respiratory rate was normal. There were no abnormal physical signs on examination of the chest. The leucocyte count was normal or slightly elevated with the percentage of polymorphonuclear cells either normal or slightly increased.

The diagnosis was usually made on the third to fifth day when roentgenogram of the chest was made and a small abnormal area of moderate density was found in one lung field. A day or two later crepitant râles could be heard at the base of one lung, posteriorly. Usually there were no physical signs indicating pulmonary consolidation. The cough continued and by then the patient had begun to expectorate a small amount of mucopurulent sputum. The temperature returned to normal in about 6 to 8 days. Defervescence was by slow lysis. By this time medium moist râles could be heard at the base of one lung and usually persisted for 7 to 10 days. The patient remained in the hospital another week or two and was returned to duty well.

ANALYSIS OF SIGNS AND SYMPTOMS

Approximately one-fourth of our cases had a relatively sudden onset, under 24 hours. Only one-sixth had a previous history of an upper respiratory infection, such as colds and sore throats, during the 3 weeks preceding the onset. Approximately one-third had typical nasal symptoms of an acute rhinitis at the onset of the infection; one-fourth had a mild sore throat. Only two patients had an actual chill and only three complained of a chilly sensation. One-fourth complained of substernal discomfort which was usually a burning sensation, although a few complained of substernal tightness. There was no actual chest pain characteristic of pleurisy; no dyspnea or the expectoration of bloody sputum.

The physical examination at the onset was characterized by its lack of abnormal findings. In approximately three-fourths of the cases the chest was normal. In eight cases there were crepitant râles at the bases of the lungs, but most of these reported to the sick bay after having been sick for a few days. In a few cases sibilant râles were present and in about the same number sibilant and crepitant râles were both heard. These were usually located at the base of the lungs, posteriorly. Diminished breath sounds were present in a few cases and dullness on percussion in only one. There were no definite physical signs of pulmonary consolidation in any case at the onset of the infection.

The temperature curve varied moderately during the course of the disease. In the majority of the cases the temperature would rise to 101°-102° at the onset, remain elevated for 4 to 8 days, dropping gradually to normal. In about one-sixth of the cases the temperature was elevated to 103°-104° for several days. These patients were definitely sicker than the others, as manifested by

associated clinical evidence. In five cases there was a low-grade fever under 100° which ran from 12 to 23 days. The pulse temperature ratio was either normal or a relative bradycardia was present.

During the course of the illness the two most significant factors were the paucity of chest findings on physical examination and the persistence of râles once they appeared. In five cases râles were heard before the third day, but in these we were doubtful as to whether the history of the day of onset was accurate. In the large majority of cases râles were heard first from the fourth to the eighth day of the illness. In one-fourth of the cases no abnormal chest signs were ever apparent and in four cases râles were not heard until the ninth to the eleventh day. The râles were usually medium, moist in type, and usually persisted from 1 to 2 weeks.

In the large majority of cases the sputum was usually absent or very scanty for the first 4 or 5 days, and after that the patient expectorated small amounts of mucopurulent material for a few days.

Approximately one-half the patients were in the hospital for 3 weeks and the majority of the remaining were in about 2 weeks. A few cases were sent to duty in 8 to 10 days; these were very mild cases. In the majority of cases hospitalization was prolonged by the weakness of the patient, a persistent cough or râles after all other symptoms and signs had subsided. The complete recovery of the patient was essential in our cases because of the necessity that he be fit for any and all military duty before being allowed to leave the hospital.

There were no serious complications in any of our cases and no fatalities.

ROENTGENOLOGICAL DATA

The outstanding observation concerning the roentgenographic findings was the fact that changes within the lung were present for several days before they could be determined by physical examination of the chest. Three of our cases had roentgenograms as early as the second day, and changes were found in all of these. In Murray's series of cases changes were found within 24 hours after the initial symptoms.

The roentgenograms usually showed a small irregular or rounded area of soft to moderate homogeneous density in the central portion with borders shading into normal lung. In many of the cases the density was very thin, although of an even quality. This was particularly true of those lesions appearing in the costophrenic angle. As a whole, the lesions were not extensive, particularly those extending from the lower border of the hilus and those found in the costophrenic angle. It was our opinion that some of the lesions extending below and outside the left border of the heart would have been shown to be more extensive could we have obtained lateral roentgenograms of the chest. The distribution of the lesion was lobular rather than lobar and the majority showed only a small section of one lobe to be involved. In a few cases the lesion was more extensive, but these were the exception rather than the rule. Multiple, successive roentgenograms in several cases demonstrated that as the disease progressed the density increased, became more sharply defined and frequently increased in size. Also, resolution was shown to follow the course of other types of pneumonia, the density gradually diminishing and becoming more mottled and linear in type.

In the majority of cases, approximately three-fourths, the lesion either extended from the lower border of the hilus region into the cardiophrenic angle, or appeared either in the costophrenic angle or at the base of the lower lobe of the lung. Sixteen cases had lesions at the left base, 7 at the right base, and 5 at both bases. Seven cases extended from the hilus toward the mid-portion of the lung. In 4 cases the lesion occurred in the upper lobe, and in 2 cases there were lesions in both upper lobes. In 1 case, lesions were located in the right upper and lower left lobes.

LABORATORY DATA

The average case showed a normal or slightly elevated initial leucocyte count and a normal to slightly elevated percentage of polymorphonuclear cells. Only 2 leucocyte counts were below 6,000 and the majority ranged between 6,000 and 9,000; 6 counts were above 10,000, but a few of these were obtained late in the disease.

Because of several factors inevitably concomitant with the commissioning of a new hospital, we were unable to carry out extensive bacteriological studies. We were unable to inoculate any animals with either the blood, sputum, or nasopharyngeal washings of any of our patients. In one-sixth of the cases we examined the sputum for tubercle bacilli and the predominating type of organism. Tubercle bacilli were not found in any specimen examined. Staphylococci, streptococci, pneumococci and Micrococcus catarrhalis were the predominant organisms found. When pneumococci were found present, we attempted to determine the type but were unable to do so except for one case which was pneumococcus type IX. The sedimentation rate was moderately elevated in the large majority of cases.

TREATMENT

The treatment was largely symptomatic with the emphasis on rest, supportive measures, and sedatives for cough and restlessness. In 12 cases sulfathiazole was administered with 30 to 90 grains given initially and 15 grains thereafter every 4 hours night and day.

We were definitely convinced that the drug was not as effective in this group as in the usual pneumococcic infections, and there was considerable question as to whether it had any beneficial effects.

SUMMARY

A series of 40 cases of an acute infectious disease of the respiratory tract with unusual pathologic changes in the lung have been described and analyzed. In general, our cases closely resemble those described as some type of pneumonitis by workers in the United States and abroad.

The majority of the cases in this series began as a simple acute infection with minimal respiratory and constitutional symptoms: a normal or slightly elevated leucocyte count and no abnormal physical signs. The diagnosis was usually made by means of the roentgenogram and *in the large majority of cases could not be made by any other means.* It usually showed a small area of increased density most often located at the base or hilus of one lung. Abnor-

mal physical signs in the lungs, usually manifested by crepitant râles, were not apparent until the peak of the severity of the disease had passed. Although the constitutional symptoms were not severe, a prolonged period of convalescence was usually required because of the weakness of the patient.

The pathology and etiology of this disease are still undetermined, although there is some evidence to indicate that it is caused by a filtrable virus.

The disease is apparently contagious, but only mildly so, and is probably transmitted by casual contact.

Lack of physical fitness, previous upper respiratory infections, abnormal exposure, or physical fatigue were not predisposing factors.

The disease did not respond favorably to the action of sulfonamide chemotherapy.

The study of our series and the reports of other workers who have observed similar outbreaks impresses us with the fact that many factors remain to be studied before the entire field of acute respiratory infections can be charted and particularly is this true of those caused by a filtrable virus.

We agree with Francis that this can be achieved only by clinical epidemiological methods and that by comparing the clinical differences in patients and epidemics with specific laboratory data, the various entities will fall into their proper places in the puzzle of epidemic respiratory disease.

BIBLIOGRAPHY

1. Allen, W. H.: Acute Pneumonitis, Ann. Int. Med. **10**: 441-446, October, 1936.
2. Ackerman, A. J.: Roentgenological Considerations of Influenzal Pneumonitis, Am. J. Roentgenol. **46**: 175-181, August, 1941.
3. Bowen, Albert: Acute Influenza Pneumonitis, Am. J. Roentgenol. **34**: 168-174, August, 1935.
4. Bullowa, J. G. M., and Wilcox, Clare: Endemic Pneumonia, Ann. Int. Med. **59**: 394-407, March, 1937.
5. Cass, J. W., Jr.: Question of Influenza and Atypical Pneumonia, New England J. Med. **214**: 187-193, January, 1936.
6. Gallagher, J. R.: Broncho-pneumonia in Adolescence, Yale J. Biol. & Med. **7**: 23-40, October, 1934.
7. Gill, A. M.: Pneumonitis, Brit. M. J. **1**: 504-507, March, 1938.
8. Francis, T. F., Jr.: Epidemic Influenza: Studies in Clinical Epidemiology, Ann. Int. Med. **13**: 915-922, December, 1939.
9. Miller, F. N., and Hayes, M. G.: Broncho-pneumonia of Mild Severity at University of Oregon, N. W. Medicine **38**: 12-14, January, 1939.
10. Marfield, J. R.: Atypical Pneumonia With Leukopenia, Texas State J. Med. **35**: 340, September, 1939.
11. Murray, M. E., Jr.: Atypical Broncho-pneumonia of Unknown Etiology, New England J. Med. **222**: 565-575, April, 1940.
12. Reimann, H. A.: An Acute Infection of the Respiratory Tract With Atypical Pneumonia, J. A. M. A. **111**: 2377, December, 1938.
13. Reimann, H. A., and Havens, W. P.: An Epidemic Disease of the Respiratory Tract, Arch. Int. Med. **65**: 138, January, 1940.
14. Scadding, J. G.: Disseminated Focal Pneumonia, Brit. M. J. **2**: 956-959, November, 1937.
15. Lusiley, D. F., Showacre, E. C., Lee, W. F., and Ferris, H. W.: Acute Interstitial Pneumonitis, J. A. M. A. **112**: 1901, May, 1939.
16. Editorial: Virus Studies in a New Respiratory Infection, J. A. M. A. **115**: 2180-2182, December 21, 1940.

Erratum

On page 269 of the December issue of the JOURNAL, in an article entitled "The Effect of Testosterone Propionate in the Treatment of Arteriosclerosis Obliterans" by Herman Zurrow, M.D., Gamliel Saland, M.D., Charles Klein, M.D., and Stanley Goldman, M.D., New York, N. Y., the footnote "†Deceased" pertains to Dr. Goldman and not to Dr. Saland.

The Journal of Laboratory and Clinical Medicine

VOL. 28 FEBRUARY, 1943 No. 5

SYMPOSIUM ON WAR MEDICINE. II.

WAR AND MEDICINE*

HENRY E. SIGERIST, M.D.

I MUST begin by confessing that I am not an expert in this particular field of medical history. My researches have been along somewhat different lines, although I have touched the history of war surgery in several previous publications.[1] Men like Colonel Fielding H. Garrison[2] and Colonel Edgar E. Hume[3] have made infinitely more important contributions on which I will have to draw heavily. I am, however, extremely interested in the subject because, directly or indirectly, wars have played a very important part in the development of medicine, and also because during the last war I was an army physician myself. Since the subject is very broad, I must limit myself to tracing briefly the development.

War's contribution to medicine is, first of all, negative. It destroys the best results of the physician's work. The elite of a nation, young men trained and nourished scientifically, immunized against a great variety of diseases, are shot to pieces, are killed or crippled. War, however, affects also the health and well-being of the civilian population adversely because it is a catastrophe that usually does not come alone. War, famine, and pestilence have at all times constituted a triad of evils. The farmer has to abandon the plow, the fields are destroyed, the granaries are looted; famine and deficiency diseases result. Transportation is upset. After the Industrial Revolution the population increased in most western European countries beyond the productive capacity of the soil, so that countries had to rely on the importation of foodstuffs. The blockade, therefore, became one of the most formidable weapons, with the result that malnutrition and famines developed. Famine and pesti-

*From the Johns Hopkins University Institute of the History of Medicine.
Read before the Washington Academy of Medicine, April 22, 1942.

lence, more than the actual war, devastated Central Europe in the seventeenth century. It was a contributing factor in Napoleon's defeat of 1812. Scurvy played a very important part in the Sixth Crusade in the thirteenth century, but also in the armies of Napoleon in Egypt and Russia, and during the World War in the British army in Kut-el-Amara.

Every war was a fertile breeding ground for the development of epidemics. Large groups of people are massed, filth accumulates, the very subtle public health machinery is frequently upset, the physical resistance of the population is reduced by malnutrition or other factors, epidemics develop, and more than one war has been lost as a result. Greece was saved from Persian invasion in 492 by the outbreak of an epidemic that decimated the Persian armies. The plague of 429, the character of which is still controversial, accelerated the downfall of Athens. An epidemic stopped the Huns before Constantinople in A.D. 425. The Black Death of 1348 had tremendous repercussions on Europe's war history. England, equipped with the new firearms, had defeated the French at Crécy and had conquered Calais after a siege of eleven months. The plague forced them to withdraw and compelled them to sign a truce. The Scottish allies of France, who intended to invade the north of England, were not only defeated by the English troops but also decimated by the plague. The kingdom of Naples was saved by the plague which forced the Hungarian army of occupation to a hurried retreat.

From the Renaissance on, typhus was one of the most dangerous enemies, ever ready to defeat brilliant armies and to play havoc with the civilian population of many countries.[4] The louse was still triumphant in the eighteenth century, and in almost every European war of the period, including the campaigns of Napoleon, more people died from typhus than from wounds. Conditions changed in the second half of the nineteenth century when the battle between soap and the louse resulted in the latter's retreat for the first time. Typhus disappeared from most western European countries, and armies could destroy each other without interference. But the disease remained endemic in eastern Europe, and we all remember the part it played in the First World War, followed by the terrific devastations it caused in Russia from 1918 to 1922. We do not know yet what part typhus will play in the present war. It seems that the disease is still under control, and it is very probable that it will remain so for some time. The actual danger will come later when the war enters its decisive phase, when armies collapse, and civil unrest develops. Then typhus may again raise its head and play its traditional role in the shaping of history.

Until very recently disease caused more casualties than battle wounds. In the Crimean War the French army of 309,000 men lost 20,000 from wounds and 75,000 from disease, a proportion of almost 1 to 4. In the short Italian campaign of 1859, which lasted only two months, the French lost 8,674 men from disease and 3,664 from wounds. In our own Civil War typhoid and dysentery killed more people than the battles. The Franco-Prussian War of 1870 to 1871 was the first to reverse the proportion, at least in the German army. Germany lost 40,881 men, of whom 28,282 were killed by weapons, 346 died from accidents, and 12,253 from disease.

In the past, health standards of the civilian population were affected primarily by the food shortages and epidemics resulting from war. In the total wars of today a new factor has entered the scene. Today wars are fought not only by armies but by nations. Industrial production has become a major weapon and requires the mobilization of a country's entire man power including women. We have no figures yet, but we can readily imagine what health conditions must be among the millions of European workers who have been dragged away from their homes and are performing slave labor for Germany.

In our own country the sudden expansion of the war industries has led to a considerable dislocation of the population. Thousands of workers live in trailers and in shacks, or sleep in "hot beds" which are hardly conducive to health. Already the number of industrial accidents has increased, and we may also expect an increase in the incidence of tuberculosis, which is likely to happen when large numbers of women are drawn into industry. Under normal conditions industrial expansion would call for added medical facilities, but under war conditions the demand cannot be satisfied because the armed forces absorb a high percentage of the available medical personnel and equipment. Thus a vicious circle results which can only be offset by a thorough organization of all medical resources in a way that guarantees maximum efficiency and minimum waste.

There can be no doubt that the major immediate contribution of war to medicine is negative. War is a destructive process which destroys what medicine has endeavored to build. But war, on the other hand, is also a challenge and a stimulus to medicine, both technically and socially. Medicine is always facing a condition of emergency, because in the fight against disease there is no armistice. War, however, is an emergency of a particular kind which puts a nation's medical resources to a severe test. It also provides opportunities for experimentation on a tremendous scale, such as would never be available in times of peace.

Of all branches of medicine, surgery obviously is the one which at all times responded to the war challenge most strongly and in various ways. Armies travel and with them go the army surgeons. They meet colleagues from foreign lands and exchange experiences. This already happened in ancient Greece when Greek surgeons traveled with the mercenaries who were in the army of the Egyptian pharaoh Psammetich. They probably learned from their Egyptian colleagues, which may explain the fact that similar operations are described in the Egyptian and Greek surgical literature. Surgery is not only transmitted through books, but, first of all, in a practical way, with experience being passed on from father to son, from master to pupil. In this process of transmission wars must have played a very important part.

War was also responsible for the erection of hospitals in ancient Rome. The Greeks never had hospitals in our sense; physicians sometimes took a few patients into the guest rooms of their workshop, the *iatreion*, but these were certainly not regular hospitals. In Rome, however, practical considerations led to the establishment of special institutions for the care of certain groups of sick people. Every soldier was a fighting force, and it was in the interest of the army to restore the wounded as quickly and as thoroughly as possible. Lazarettos were, therefore, built, and the foundations of several of them have

been excavated, particularly in the periphery of the Empire. Some of them, like the one in Novaesium, were very elaborate institutions with many sick-rooms opening into large corridors built around a vast courtyard.[5] The military hospital in Baden, in Switzerland, was close to a sulfur spring, and extensive use was made of the medicinal water in the treatment of the wounded.

War, or rather the lack of war, caused the establishment of another kind of Roman hospital, the slave *valetudinaria*. Slaves were cheap as long as wars guaranteed a steady stream of prisoners. But the value of slaves increased considerably once the empire was pacified, and it became profitable to spend money for the restoration of the slaves' health. Thus slave hospitals were erected on the large estates of Roman landowners.

Until the fourteenth century most war wounds were caused by swords, spears, arrows, and similar weapons. They, as a rule, healed by first intention, and in the writings of thirteenth century surgeons wound healing without suppuration was considered the normal process that a physician should strive to attain whenever possible. Conditions changed when firearms were introduced in the fourteenth century. The wounds caused by the large caliber lead bullets of those days were primarily infected, and the view was generally accepted that gunshot wounds were poisoned by gunpowder and had to be treated with the cautery or by pouring boiling oil into the wound. It is well known how a military surgeon, Ambroise Paré, the father of modern surgery, refuted this view on the basis of experiences gained on the battle fields during one of the campaigns of Francis I against Charles V. He proved experimentally that gunshot wounds healed much better without the brutal treatment that destroyed so much of the tissues. But even to Paré suppuration appeared as the normal wound-healing process. Paré's treatise on the treatment of gunshot wounds was published in 1545 and revolutionized war surgery. Thereafter he published a large number of other surgical books, and frequent campaigns in which he participated gave him an opportunity to test his operations on a large scale. He reintroduced the ligature of arteries when performing amputations, was also a pioneer in the field of obstetrical operations, and exerted a tremendous influence in the whole field of surgery.

When John Hunter took part in the expedition to Belle Isle in 1761, the British navy availed itself of the services not only of a surgeon but also of a scientist. On this expedition Hunter gained invaluable experience on gunshot wounds, a subject that he studied all his life and which he finally discussed in his masterpiece, *A Treatise on the Blood, Inflammation and Gunshot Wounds,* that was published in 1794 soon after his death. In those days, most surgeons were still craftsmen who were primarily interested in the practical side of surgery. Hunter was a surgeon also, but a scientist in addition. For him a wound was something more than a practical problem. He was not content to ask, "How can I best heal this wound?" He inquired, "What does the wound signify to the organism? By what mechanisms does the organism safeguard itself against the effects of the wound, immediate and remote?" In this way, almost imperceptibly, he passed from the domain of surgery into that of pathology. The main significance of his work was that he threw open the field of surgical observation and experiment to general medicine. He was the first investigator

since the days of antiquity to advance the theory of inflammation, and there can be no doubt that Hunter's war experience had a great influence on his later researches.

Until the middle of the nineteenth century, the field of surgery was limited to a number of classical operations which had been gradually improved from century to century. Major abdominal surgery was impossible as long as one could not operate on the relaxed body, and also on account of pain and secondary infection. The introduction of general anesthesia in 1846, and of antisepsis in 1867, when Lister's first publications came out, liberated surgery from these age-old bonds. Both methods were immediately applied in war surgery and undoubtedly helped to save thousands of human lives. In 1846 Warren performed his historic operation at the Massachusetts General Hospital, while Morton gave the anesthesia, and the following year Syme applied general anesthesia in the Mexican War and Pirogov did the same in Russia. In 1867 Lister published his first papers, and a few years later the Franco-Prussian War provided an opportunity to apply and test the antiseptic treatment of wounds on a large scale and to gain much experience in the field. The German surgeons, Volkmann, Thiersch, and Mikulicz, were particularly active in developing and popularizing the method.

The Franco-Prussian War also became the testing ground for the efficacy of smallpox vaccination. In the German army, where most soldiers were vaccinated and revaccinated, 4,800 cases of smallpox occurred with 278 deaths, while among the unvaccinated French prisoners alone 14,000 cases occurred with 2,000 deaths.

All fields of medicine felt the challenge of war, surgery more than any other, but also public health was stimulated. Problems of sanitation were particularly acute in times of war, and in the seventeenth and eighteenth centuries many books were written on the sanitation of camps and barracks and on general problems of military hygiene. In 1738 a great soldier, the Maréchal de Saxe, wrote a book with the title, *Rêveries ou mémoires sur l'art de la guerre.* It was the result of thirteen nights of insomnia when he was suffering from a fever disease, not a textbook on strategy, but a thoughtful book on military hygiene. Uniforms were more spectacular than convenient. They were devised to satisfy the vanity of the ruler and to make the soldiers attractive to the fair sex as a compensation for the professional risks they ran. The Maréchal de Saxe advocated a different type of uniform better adapted to the purpose they had to serve. He also gave much thought to the soldiers' diet. Long before Liebig, in the middle of the eighteenth century, meat extracts were prepared to feed soldiers during campaigns.[6]

War had repercussions not only on medical science but also on the humanitarian side of medicine. Florence Nightingale would have become the reformer of nursing without a Crimean War, but there can be no doubt that the experience she gained at Scutari in those terrible years 1854 to 1856 accelerated developments tremendously. October 21, 1854, the day when she left England with 38 nurses, truly marks a turning point in the history of nursing. Placed all of a sudden under conditions of great emergency, Florence

Nightingale saw and did more in less than two years than she could have done in a lifetime in England. The Crimean War thus actually became the cradle of modern nursing, just as the Battle of Solferino in 1859 became the starting point of the Red Cross. The Geneva banker, Henri Dunant, who was visiting Italy as a tourist, suddenly found himself on the battlefield of Solferino and was so stirred by what he saw that he devoted his entire life to the alleviation of the sufferings of the wounded and prisoners of war. In October, 1863, 62 delegates, representing 16 nations, convened at Geneva, and the Red Cross Society was founded the following year. As we all know, it has extended its scope far beyond war activities and in every country is playing a tremendous part in relieving human suffering.

Ambroise Paré and John Hunter, whom we mentioned before, served as army surgeons, but only occasionally. Paré was surgeon-in-ordinary to several French kings, and it was obvious that he accompanied them whenever they went to war, but otherwise he practiced surgery as a civilian in Paris. John Hunter's military period was only an episode in his life, although an important one. Great contributions have been made to medical science, however, by medical men for whom the army was a career, and there can be no doubt that no medical corps in the world has done more for the advancement of science than that of the United States Army. It is impossible to list all these contributions, and all I can do is to recall a few names: Beaumont who experimented in the wilderness on the gastric juice; John Billings whose contribution to hospital construction, medical education, vital statistics, and the development of libraries has been unsurpassed; the heroic work on yellow fever of Sternberg, Reed, Carroll, Lazear, Agramonte; Gorgas whose activities extended far beyond the Panama Canal and who was instrumental in organizing medical services in the gold mines of South Africa; Ashford's studies on hookworm, Craig's on malaria, Woodward's on photomicrography. Colonel Hume has recently shown in a scholarly publication what great contributions army surgeons made to the science of ornithology.[7] There is hardly a field of general or medical science that has not been enriched by members of the United States Army Medical Corps, and the Army Medical Library in Washington with its splendid collections and bibliographies is a unique institution that has played an important part in the development of American medicine.

There is one thing we can learn from history, namely, how tremendously important the medical corps is. There was a time, not so long ago, when the generals used to consider the medical officer a nuisance, the man who always interfered with their dispositions. They forgot how many campaigns were lost in spite of brilliant strategy as a result of diseases. Views are changing rapidly, and the war departments are recognizing the need for mobilizing all the resources of medical science.

Medicine learned from every war, and the more science progressed the more medicine became able to save human lives even in war. The soldiers who today are sent to foreign lands are immunized against many once fatal diseases; they are nourished scientifically and they carry along the vitamins needed to protect them from deficiency diseases. They are clothed infinitely

more hygienically than in the past. Operative results have been greatly improved, and the new methods of blood transfusion are saving countless lives. The great progress achieved by orthopedics makes it possible to rehabilitate the wounded infinitely better than in the past. In previous centuries little attention was paid to crippled war veterans. A few were assigned to the care of monasteries, but the great majority became beggars roaming the streets. It was Louis XIV in France who established the *Hôtel des Invalides* in Paris, an institution in which invalid veterans were attended at the expense of the state.

The rehabilitation of the crippled veterans is one of the most important social problems of war medicine today. Pensions alone do not solve the problem. Work is one of the major balancing factors of health, and there is plenty of skilled labor that can be performed by blind and maimed veterans. Every effort must be made to readjust the invalid to his environment so that he can continue to live as a useful member of society and thus preserve his self-respect. The Soviet Union is handling the problem in an admirable way. Special schools have been established for the purpose, giving general and vocational training in courses that may last as long as two years. Invalids who had only elementary education may improve their general knowledge by taking courses of secondary education. Vocational training produces bookkeepers, technicians, agricultural experts, tailors, photographers, telegraph operators, etc. The Commissariats of Social Welfare are in charge of rehabilitation programs. Local social welfare bodies must place invalids within two days, and all industrial and other enterprises are urged to provide suitable work and housing for the veterans, who are visited by special commissions every month.

War is not a natural catastrophe or a biological process, as has sometimes been said. It is man made and represents a primitive method of conducting international politics. Civilization is a very young phenomenon in the history of mankind, so that we should not be astonished that relapses into primitive savagery still occur. There is no reason, however, why we should not be able to develop in the course of time from a competitive to a cooperative society on a world-wide scale. As long as wars occurred they always had definite results that affected social and economic conditions of the countries involved in a way that was either detrimental or beneficial to the development of medicine.[8]

The campaigns of Alexander the Great propagated Greek culture all over the ancient world and created conditions favorable to the development of medicine in such countries as Egypt and Syria. The Roman conquest of western Europe pacified the Germanic tribes, developed trade, and permitted cultural developments that also benefited medicine. Persian medicine flourished as never before after the Arabic conquest, and Persia became an important province of Islamic civilization.

The Thirty Years' War, on the other hand, was a strongly retarding factor, not only on account of the destruction it brought all over central Europe, but also because it perpetuated feudalistic trends. The emergency created by the revolutionary wars of France greatly accelerated the reorganization of medicine initiated by the revolutionary government. The reforms were continued by Napoleon and became the foundation upon which the French school developed and flourished. The Franco-Prussian War liberated France from

the rottenness of the Second Empire, and led to the establishment of the Third Republic under which science and medicine reached great heights. The World War of 1914-1918 stimulated medical science in many ways as we all remember. Yet the contribution of that war to medicine was probably still greater in the field of social medicine. The war had demonstrated the importance of protecting the workers' health, and industrial medicine developed as never before. Workmen's compensation was introduced in a number of countries, and general health insurance in France. Various countries established ministries of health, and the Soviet Union made a totally new departure in creating the type of medical organization best adapted to the new industrial society and the new technology of medicine.

In the United States the examination of almost three million drafted men revealed that 47 per cent were defective; in other words, that health conditions were not as good as they could have been and that there was a maladjustment in the distribution of medical care. The Committee on the Costs of Medical Care was founded. Other agencies joined in the work, and for ten years surveys were made that provided us with the facts and figures needed for intelligent planning.

The war in which we are engaged at present will undoubtedly again stimulate medical science, but I am convinced that its social effects will be much more strongly felt. It would be foolish to assume, as some people do, that Europe is finished. Europe is going through one of its periodic crises, and the very destruction will force the European nations to reorganize their social and economic structure from the bottom. Thus conditions may well be created that will enable medicine to develop more rapidly and more broadly than in the past.

The war is demonstrating to us that our technology has outrun our sociology. We have created ingenious machines, but not the social and economic organization that an industrial society requires. We have built means of transportation that overbridge the continents, but not the apparatus that insures peaceful cooperation between nations. The same happened in medicine, and now, under pressure of the emergency, we have to organize medical services, not only for the armed forces but for the nation. If we learn a lesson from this experience, it may well become this war's greatest contribution to medicine.

REFERENCES

1. The Book of Cirurgia by Hieronymus Brunschwig, with a study on Hieronymus Brunschwig and his work by Henry E. Sigerist, Milan, 1923.—Ambroise Paré, Die Behandlung der Schusswunden, eingeleitet, übersetzt und herausgegeben von Henry E. Sigerist, Leipzig, 1923.
2. Notes on the History of Military Medicine, reprinted from The Military Surgeon, 1921-1922, Washington, D. C., 1922.
3. Medical Work of the Knights Hospitallers of Saint John of Jerusalem, Baltimore, 1940, and many other publications.
4. Zinsser, Hans: Rats, Lice and History, Boston, 1935, Little, Brown & Co.
5. Meyer-Steineg, Theod: Kranken-Anstalten im griechisch-römischen Altertum, Jena, 1912. (Jenaer medizin-historische Beiträge, Heft 3.)
6. [illegible] essés à travers l'Histoire, Paris.
7. [illegible] States Army Medical Corps, Baltimore, 1942.
8. [illegible] of the Effects of War on Medicine, Ann. Med. History, Third Series, 4: 189, 1942.

BACTERIAL WARFARE

The Use of Biologic Agents in Warfare

Colonel Leon A. Fox, M.C., U. S. Army

Editor's Note.—This article was first published by Colonel Fox, then Major Fox, in March, 1933. It has been republished and quoted extensively in technical military periodicals. It was republished in The Military Surgeon in May, 1942. The author states that, while some advances have been made since the original publication of this article, the principles are as applicable today as when first stated.

BACTERIAL warfare is one of the recent scare-heads that we are being served by the pseudoscientists who contribute to the flaming pages of the Sunday annexes syndicated over the Nation's press. This question of bacterial warfare has been brought forward from time to time since the World War. The use of the organisms that cause communicable diseases as an instrnment of warfare was considered by the Conference on the Limitation of Armaments held in Washington in 1922. An international commission consisting of Professors Pfeiffer (Breslau), Bordet (Pasteur Institute), Madsen (Copenhagen), and Cannon (Harvard), appointed at the time, reported to the League of Nations essentially as follows: (a) The effects of bacterial injury cannot be limited or localized. (b) Modern water purification methods protect against the organisms of typhoid and cholera. (c) Plague is a disease that would be as dangerous for the force using the organisms as for the attacked. (d) The danger from typhus has been exaggerated. (e) Modern sanitary methods are effective in controlling communicable diseases.

Following this pronouncement by these eminent scientists, the question of bacterial warfare suffered a lapse of interest; but during the past year, as an incident of the preparation for the Geneva Convention, there has been a marked revival of interest in this supposed bugbear, bacterial warfare. Possibly this is only a part of the effort of professional pacifists to add all the imaginary frightfulness they can picture to the known real horrors of war.

The space and thought that have been given to this question by feature writers have not been without effect, and many people now believe that bacterial warfare represents a real threat and problem for future generations. Many are now associating chemical warfare and bacterial warfare, with the result that in the resolution of adjournment, voted by the General Commission of the Disarmament Conference on July 23, 1932, at Geneva, we find chemical, bacteriological, and incendiary warfare grouped for consideration. The mere fact that this great body of peace workers considers bacterial warfare seriously enough to prohibit its use justifies military men in considering this agency of

Reprinted from The Military Surgeon 90: 563, (May), 1942.

warfare. We know how little treaties protect; so we should study the question to see if the use of biologic weapons is a real problem for the military minds of the future.

Under biologics we include all those organisms that may invade the body of man or animal to produce disease, so that while we use the term bacterial warfare, we do not limit this paper to a consideration of bacterial diseases. We will also consider the filtrable viruses, Protozoa, and other pathogenic forms as well as their toxic products.

With the powers of the world in session at Geneva discussing the future of warfare, and with some of the great nations of the world recommending the complete abolition of chemical warfare, it may appear strange to have one consider biologic warfare. I believe all will agree that while it is a mistake to live in the past, it is equally undesirable to ignore the lessons of the past in prognosticating regarding the future. It is therefore well, before we consider the possible use of biologics in warfare, to discuss briefly the question, Will the nations of the world abandon the use of chemicals as an instrument of warfare?

Every advance in thought or design meets reaction and antagonism from the minds of the previous generation. It does not take some radical departure from the accepted views of the day, such as marked the revolutionary concept of Copernicus or Darwin, to start all "as is" conservatives on a tirade of opposition with the usual tenor of their remarks: "It is against the law of nature," "It is against religion," "It conflicts with all known law," "Even if true, it does not fit into the existing order of things." A man of middle age today may remember the old mossback who refused to ride on the train of the nineteenth century. In fact, the train and street car had not completely overcome all the pooh-poohs of the backward ignoramus until they were involved in a fight for their very existence with a newer means of transportation—the motor. The motor vehicle had just had time to have a proper road net constructed when this engine, becoming "air-minded," needs no roads. Have they been generally accepted? Certainly. However, remarks such as, "If God wished man to fly he would have given him wings," were made in the pulpits of this country during the present century.

It takes more than the harpings of the minds of yesterday to scotch the wheels of progress. It may startle many to talk of world progress in connection with implements of warfare. However, it is not believed that any fair-minded individual can deny the place in world advancement that is due to the spirit of conquest. The peaceful shepherd, content to watch his flocks, has added little to the world's knowledge. The trader and warrior have discovered and spread knowledge. Trader and warrior are almost inseparably associated throughout history, and slowly as they may have progressed, they usually lead the thought of the day. The spirit of adventure and discovery has always marched with the warrior. The discoveries of the warrior are not limited to implements of war; however, these are the factors we wish to consider. In this field again we meet the same antagonism at every advance that the fighting man has made, an antagonism that has affected all minds of the "as is" type, including conservative and reactionary individuals. Every advance, every discov-

ery of a new weapon by the fighting man, has had to overcome two groups of opponents: (1) The fixed and established military group that is always sure the new weapon "won't work," "Is not as good as older weapons," "Not practical," etc., (2) The pacifist group—the shepherd group, that considers each new weapon more terrible than the former and cries out against it.

Primitive man in his combats certainly had no weapons. Are there any today who believe that this early creature did not fight over "food and females?" It may be added that all combat ultimately resolves itself in the final analysis to a strife for one of these basic biologic requirements—nourishment or sex. Fighting over gods was a later development; and these fights over gods were over a personal God, a God of the land or tribe, a God to favor their own special country, a benevolent God who would make their country a more bountiful place to live.

In the early combats man could only bite and claw and choke an adversary. This was the day of brute strength. Cleverness had relatively little value. The first man to use a weapon was the man with the best mind of his day. The first weapon used must have been very simple and elementary. Possibly a hard object held in the hand with which he brained his opponent. This weapon possibly did not create much comment. This was not an age of comment; however, the descendants of the type that could not learn to use this weapon are not numerous. Has this weapon been abandoned? Certainly not. It is an excellent weapon, and no good weapon has ever been discarded. Its use today is very limited because of the discovery of other weapons of greater range and effectiveness.

Development of weapons has always been for the purpose of using intelligence to overcome mere physical force. The factor of range, killing an opponent before he can close with you, is a most important factor when the man of intelligence must meet superior physical force or number. Probably the first weapon to provide range was a club, possibly a sharp stick, the forerunner of the iron-tipped spear. The club may have had a stone head attached. These weapons not only advanced the clever man over the mere strong man; they aided man in his fight with the man-eating animals of the time. However, if we can make deductions from the early cave records of men of this period, advance was slow because the intelligence was of such low order that they were slow to understand and accept these new weapons. The race improved because the thinker, the successful warrior, lived and won the females and left descendants; the slow and reactionary type did not live to reproduce. With every advance in weapons man is giving evidence of a desire to overcome brute strength by means of a weapon with range and effectiveness.

We can picture the introduction of the early propelled instruments, such as the arrow, causing a storm of opposition. Some youngster designed some form of propelling instrument for a sharp stick and possibly suffered the jeers of the snaggled tooth elders as he shot the sticks into inanimate targets, and received only the reward of complete recognition when he shot a sharp stick through the belly of an old pack leader to take over a band of cowed females. The progeny of this genius were of a higher order of mentality and possibly soon learned the

value of organization, with the result that a tribe of arrow users developed. This seemed like the final advance, and who can doubt their ability to inflict their will on the men of the time.

The fact that the conquered men, possibly of superior physical development, considered the weapon a cruel and brutal implement that God had not endowed man with did not cause it to fall into disuse. The only thing that caused this weapon to fall into disuse, and finally be practically abandoned, was the development of such protection as caused the implement to cease to be effective or because other instruments were designed of greater range and effectiveness. These factors are the only things that have ever caused a weapon used successfully to be abandoned.

The outcry against the use of chemicals seems to people of this day to be quite a serious factor, and some wonder if their use will be curtailed by this influence. The following factors should be considered before we make a decision: (a) No effective weapon once introduced has ever been abandoned until it was displaced by a more effective weapon or protection was developed that rendered the instrument useless. (b) The hue and cry that attended the introduction of chemicals are not unusual on the introduction of a new weapon. The early use of gunpowder produced a reaction in every respect similar to the cry of the present day pacifist against gas. Will the use of chemicals in warfare be abandoned? Probably not. Will the use of chemicals be curtailed? Certainly; just as certain as the race progresses, just as certain as new and more effective weapons are designed—not before this advance is made.

Will the next advance in warfare see the use of biologics? Will the next agent used be the living organisms, bacterial warfare, the scourge of armies from the most ancient times—the communicable diseases? The question of biologic warfare will be considered in more detail because here again we run into the most elaborate and fanciful statements.

A review of military history will reveal the great influence that disease has played in past wars. Results have been decisively influenced in many campaigns by epidemics of communicable disease. In some campaigns they have caused such tremendous losses and such great numbers of noneffectives that the combat has reached a stalemate. However, in certain instances, for unknown reasons, there has been a great difference in the degree to which combatants have reacted to the epidemic conditions. In a few cases we are able to understand why the communicable diseases appeared to have greater invasive power toward one of the armies; in other instances we do not understand clearly why there was a difference in the degree of involvement of the forces.

Volumes have been written on the epidemic diseases that have attacked the military forces. We will not attempt to review this extensive literature, but the doctor, especially the epidemiologist, knows that the student of history who reads only of tactics and strategy, the victories and defeats of a campaign, without familiarity with the medical history of the war, is likely to give some commander credit for success or failure that all too often has been caused by some epidemic of communicable disease. This is not meant to depreciate military success, for

the great general is often a great sanitarian, and even Alexander may owe a part of his success to his doctor, philosopher, teacher—Aristotle's advice to "boil his water and bury his dung."

We must remember that we can march through the pages of military history all the way to the twentieth century before we come to a campaign where the missiles of the enemy produce more casualties than epidemic disease. In most of the ancient campaigns of any duration some one of the great military plagues did more to decimate the military forces than all the man-made munitions. I say one advisedly, although often many infections raged and famine and scurvy accompanied the communicable diseases.

What was the nature of these ancient pests? Were they diseases of that age now no longer known? No, the military pests that existed then are still with us. The Big Six of all time (wartimes) are: (1) the enteric fevers, typhoid and the paratyphoids; (2) the dysenteries; (3) cholera; (4) typhus; (5) the plague, bubonic plague, the Black Death; (6) smallpox.

Do not consider for a moment that the above diseases had any monopoly on the right to destroy armies. It is probable that at times influenza and the epidemic pneumonias took such heavy toll that only little fuel was left to be consumed by the Big Six. Again, under conditions where malaria is endemic, this disease is second to none in the production of noneffectives in military ranks. In fact, measles and epidemic meningitis may well be added to the list of military scourges.

This paper is not for the purpose of considering the epidemic conditions of the armies of the past, but it is realized that many individuals will naturally consider that if these infectious agents were able to produce such frightful outbreaks of disease by the simple process of chance infection under natural conditions, then in the hands of man, as a military weapon, they may well prove even more destructive. They may fail to consider the fact that the same measures that are now so efficacious against the chance infections occurring in nature may prove of equal value in combating the same agency of destruction when used by man.

We have presented biologic warfare in all its horrors; now let us analyze the problem in detail. What agents can be used to produce death and disease? How can these agents be introduced into the bodies of the enemy? We will discuss these questions in the order stated. The biologic agents available for warfare are: (1) the communicable diseases; (2) other infective processes (such as wound infections); (3) toxic products of bacteria.

The communicable diseases are well known. They are the so-called transmissible diseases that produce epidemics. They are caused by a living contagion and are spread from man to man or animal to man by various channels of transmission. All of the Big Six and the other diseases mentioned above belong to this group.

The second group, the other infective processes that are available, include such infective materials as the agents that infect wounds: gas gangrene, *tetanus*, anthrax, and other wound contaminations that are infectious but not communicable.

The last group of dangerous agents are the *toxic* products of bacterial growth. We will mention but a single terror-inspiring example—botulinus toxin.

A portion of this toxin almost inconceivably small when introduced into the body by any channel is lethal. We will give details later.

No one will question the effectiveness of all these agents in producing casualties when introduced into the bodies of unprotected and nonimmunized individuals. The important question then is how? How are these agents to be introduced into the bodies of the enemy to produce casualties?

Any consideration of the deliberate use of pathogenic organisms as a means of warfare will have to consider the question of how to produce a destructive epidemic in the forces of an opponent and at the same time protect one's own forces from invasion by the virulent organisms in question. Certainly at the present time we know of no disease-producing microorganisms that will respect uniform or insignia, and the use of bacteria in warfare for the destruction of opposing forces will have to be predicated upon the successful prior immunization or the complete isolation of the forces employing the disease-producing organisms through some system of quarantine.

Any intelligent discussion of bacterial warfare must certainly give detailed consideration to the question of how the living contagion is to be introduced into the individuals that are to be infected. We can well begin this investigation by a study of the channels of infection. The communicable diseases may be classified on the basis of their "routes of transmission." By this is meant the path that the living contagion follows when it leaves the body of the sick man or animal, or in some cases the carrier, to enter the body of the susceptible host to produce disease. On this basis we may classify the communicable diseases into intestinal diseases, respiratory diseases, direct contact diseases, and insect-transmitted diseases.

The intestinal diseases are produced when some small portion, usually a microscopic portion, of the material from the intestinal canal of the sick man with its living microorganisms, is introduced into the alimentary canal of the susceptible individual. Typhoid, cholera, and dysentery are well-known examples of this type of disease.

The respiratory diseases, sometimes known as "sputa-borne" or even "air-borne" diseases, are the communicable diseases spread by the transmission of living microorganisms from the respiratory tract of the sick to the respiratory tract of the invaded. This group of diseases is of tremendous importance and embraces such conditions as the common cold, influenza, pneumonia, diphtheria, epidemic meningitis, smallpox, and possibly of special importance for war purposes, the pneumonic form of bubonic plague.

The group of diseases that we refer to as "insect-transmitted" are those where the invasion of the new host is effected by the bites of insects which have previously fed on an individual—man or animal—infected with the disease in question. A period of incubation on the part of the insect between feedings on sick and feedings on individuals to be infected is necessary in certain instances; with other diseases such interval is not required. Examples of insect-transmitted diseases that require an interval for the development of the contagion within the body of the insect after feeding on the infected individual are malaria and yellow

fever, both transmitted by mosquitoes. Bubonic plague, a disease of rats that is transmitted to man by the bite of the rat flea, does not require an incubationary period for the rat flea to develop infectiveness.

The venereal diseases are direct contact diseases. They are of profound military importance and have proved decisive factors in certain past wars, notably influencing the European campaigns of the fifteenth and sixteenth centuries. The deliberate use, however, of this means of injury is fraught with difficulties when we plan a method of securing personnel to effect the necessary exposure. The soldier's danger from the venereal diseases will not come from the open, avowed wartime enemy who loves him least, but from the money-loving or uniform-worshipping ladies who profess to love him most. Therefore, while these diseases may at times exceed all other causes of military noneffectiveness, we can dismiss them without further discussion while we are considering bacterial warfare.

It follows, then, that the communicable diseases that constitute an epidemic or pandemic threat to the military forces are the intestinal, respiratory, and insect-transmitted diseases.

THE INTESTINAL DISEASES

Mankind is all too familiar with the terrible epidemics of typhoid, cholera, dysentery, and the diarrheal conditions that have destroyed military forces in the past. However, it is highly questionable if this group of diseases will ever in the future cause any such terrible catastrophes, for the reason that the epidemiology of these infections is so thoroughly understood that modern sanitary methods and immunization processes have rendered comparatively innocuous these hazards of earlier armies.

The deliberate use in warfare of these agents, however, we shall consider. While occasional small outbreaks of these diseases may be due to food infections, real epidemics of this group of diseases are traceable only to infected water and milk supplies, or to such a complete sanitary breakdown that general fecal contamination of food supplies occurs. The possibility of contaminating a milk supply presents practically insurmountable difficulties, although it is theoretically possible that spies might use such a means to discommode and harass civil populations. Of course, it has no practical application to the military forces themselves.

Contamination of water supplies of civilian communities by means of infection of large reservoirs and storage basins where the water is held awaiting consumption is a possibility. Contamination, to be effective, would have to be subsequent to treatment by the modern water purification plant consisting of filtration and chlorination, or, of course, it would be valueless; but this is within the range of possibilities, and it is possible that future wars will reveal that spies will make an effort to contaminate municipal water supplies.

The use of the intestinal group of diseases against forces in the field would probable prove entirely ineffective because modern water purification methods and the close supervision of the water supply that is accepted as a necessary incident of military service will absolutely preclude the successful employment of this means of combat.

In considering the intestinal group it may be well to stress the fact that the reason modern armies, and for that matter all civilized communities, do not have serious epidemics of these diseases is not that the infective agents that cause these diseases are not present or available, but that modern sanitation protects the personnel.

Let us take a typical example, typhoid fever. The incidence of typhoid in our civil population has been greatly reduced during the present century. Let no one think, however, that this is due to any scarcity of the typhoid bacillus, and it must also be remembered that the civil population has not had any general immunization such as helps to protect the Army. Typhoid has not retreated to the outskirts of civilization: it is all about us. Every state, yes, every county in the Union, is infected. Typhoid carriers in the United States possibly number 100,000 and are generally without supervision. The reason we have only about 5,000 deaths from typhoid fever per year in the United States of America instead of about 100,000 deaths is that the great mass of our people now use water that has been rendered safe by filtration and chlorination. They consume milk that has been pasteurized and other foods that have been protected.

The same statement may be made concerning the low incidence of the dysenteries in our country. The infection is present, but epidemics do not occur because our sanitary measures are effective. We need not fear infection from without with this group of diseases; we are already grossly contaminated.

The die-hards will say that cholera is not so easily handled and is not at present a problem in America. Granted. We do not have cholera in the States; but our Army and our people do live in the presence of cholera without having epidemics of the disease. The Philippine Islands, where our Army maintains an effective fighting force entirely free from this terrible scourge, has a carrier incidence of the vibrio that causes cholera that is always high. The intestinal group of diseases will certainly not prove destructive against any civilized nation that cares to pay the price of the protection that modern sanitary methods provide.

THE RESPIRATORY DISEASES

In leaving the intestinal group of diseases we proceed from the problem that represents the greatest triumph in preventive medicine to the group of diseases that baffles the best efforts of all health workers. In the control of the intestinal diseases we have so much to be proud of. In preventing the respiratory diseases we have accomplished so little. This is stated with a full knowledge of the wonderful results that have been obtained with smallpox vaccination, and the immunization to diphtheria by the use of toxin products, as well as with a full realization of the fact that we are on the threshold of equally great accomplishments in controlling scarlet fever.

It should be noted that these great accomplishments are not sanitary triumphs, such as glorify our work with the intestinal group of diseases, but immunization processes. Not being able to prevent the infection reaching mankind, we take advantage of the fact that familiarity with the organism, while not breeding contempt, does produce immunity. Therefore, we use the only method that appears to offer any great protection against the respiratory dis-

eases in nature, namely, immunization. It must be admitted that health workers can accomplish practically nothing in the way of protecting peoples from infection with the great host of respiratory invaders, and such protection as we have is due to either natural or artificial exposure to these organisms.

In this group of diseases we find a number of maladies that are serious enough to be effective war weapons if ways of using them can be devised. However, before proceeding we should call attention to the fact that in this group are also a large number of diseases that are not suited for military purposes. For instance, smallpox, while a very serious epidemic disease, must be dismissed immediately. All military forces are immunized to this dreadful scourge, and we can, therefore, dismiss it from further consideration.

Many of the diseases of childhood, while constituting a military problem at time of mobilizing rural recruits, are not suitable for military purposes for the reason that the factor of age susceptibility plays so much importance when we consider the entire group that comprises our population. As an example we may mention diphtheria. While in childhood a very high percentage of the population is susceptible to this disease, the great majority of these same individuals develop considerable natural immunity to the organism that causes diphtheria without further interference than the normal aging. Therefore, while we see epidemics of diphtheria in schools and orphanages, we do not encounter serious outbreaks involving large numbers of any adult population. This disease is cited only as an example wherein the factor of age susceptibility is important; there are a number of diseases that show this phenomenon and would, therefore, be unsuited as offensive military weapons.

Certain conditions such as influenza, pneumonia, and the common cold do not show a marked tendency to limit their injury to any one age group and would be efficacious if they could be used against military personnel. Mankind is as helpless today as at any period in history in the control of these diseases; also they are very serious conditions that produce great numbers of noneffectives, and in the instance of the epidemic pneumonia they result in a tremendous mortality.

Before we surrender to the individuals who threaten such frightful havoc with this group, we may well ask how are they going to start an epidemic of influenza, pneumonia, or the common cold. If they answer that they will introduce the germs that cause these diseases, we can well laugh at them. The process is not so simple. The factors that make respiratory epidemics are not so elementary. They include not only the infection of the individual, but the question of the resistance of the infected animal. The organisms that cause these diseases are all about us. They are always with us. Epidemics mean more than simply infection; they mean the rapid transfer from individual to individual of these infective agents. They mean a lapse in the immunity of the invaded, and possibly something else.

I do not know of a bacteriologist or an epidemiologist who can tell you how to start a respiratory epidemic unless the stage is especially set. I know many who are certain that whenever you place a large group of individuals, man or beast, under poor hygienic conditions, with overcrowding, poor ventilation,

and exposure to unfavorable climatic conditions, or other factors that decrease resistance, respiratory outbreaks will occur in spite of any precautions that can be taken, and that if large numbers of highly susceptible individuals (rural populations) are present, the outbreak can be expected to assume epidemic proportions.

It is also worthy of note that when epidemic conditions prevail, certain organisms may possibly have greater invasive power, for then apparently populations that were not so susceptible or readily invaded may be attacked when they previously escaped injury. It will be noted that as in the case of the intestinal diseases, so with the respiratory diseases it is not a simple case of introducing infection that constitutes a menace. The organisms that produce most of these diseases are always with us, and epidemics mean more than infection. While we cannot understand exactly how epidemics start, and we question the ability of a military agency deliberately to produce an epidemic of one of these diseases, we feel certain that if bacterial warfare is ever contemplated, they will not think of using the respiratory group of invaders for the reason that quarantine, isolation, and all other methods to control diseases such as influenza, are practically valueless. The torch once set off might destroy friend and foe alike, and would, therefore, prove of no value as a military weapon.

The two diseases in this group that are most frequently mentioned are influenza and epidemic meningitis (cerebrospinal fever), possibly because of their importance during the World War. All that has been stated above applies with especial force to influenza, where in addition to the fact that no one knows how to control this disease, we must add that we are not even positive about the actual organism that causes the condition. Epidemic meningitis, on the other hand, is a very definite, specific disease due to a very well-known organism. We must admit at the outset that this is a very serious disease, and that it often assumes epidemic proportions in military organizations. However, if we stop to consider the nature of the organism and the epidemiology, we see how entirely unsuited epidemic meningitis is for use as a military weapon. The organism, the micrococcus of Weichselbaum, is so delicate that even on the most favorable culture media it rapidly dies when exposed for even a few hours to temperatures much below that of blood heat. This disease is spread by carriers, and the organism must be introduced almost directly from the nasal pharynx of the carrier to the respiratory mucous membrane of the individual invaded or it will be destroyed by the unfavorable temperature conditions while en route.

Those individuals who think this disease may be used for military purposes will answer that carriers in the forms of prisoners, etc., would be introduced into the opposing forces. To those who know anything about epidemic meningitis this suggestion is ridiculous. Any military aggregation of any great size already has so many carriers present (anywhere from 2 to 30 per cent) that the introduction of a few more or less is of no moment. Epidemics of meningitis occur only when *overcrowding* is associated with conditions that lower the general resistance, as exposure, unfavorable climatic conditions, and fatigue. Meningitis is, and probably always will be, a military problem; but the individual's friends and associates, not the enemy, are the great problem with this disease.

We will not take up in detail all of the various respiratory diseases. The tabulation would prove tiresome, for the story would always be not so much a question of the great danger of the introduction of the infective agent, but the creation of epidemic conditions, a soil in which the organism could produce an epidemic, overcrowding, and lessened resistance.

THE INSECT-TRANSMITTED DISEASES

These diseases will probably most certainly influence wars of the future as they have in the past. An invasion of such a country as Mexico, at the present time, could constitute more of a sanitary than a military problem. With malaria, dengue, and possibly even yellow fever along the seaboards, and typhus endemic in the plateau district, our main problems would be sanitary. Bubonic plague might also be encountered here as well as in any other place. This disease, bubonic plague, is the disease entity that many consider best suited for military purposes. To begin with, it is a frightfully serious malady, a decimating disease that has most profoundly influenced warfare in the past. It is possible that the rise of the Mohammedan world was due to a great extent to the fact that Europe was in the throes of the greatest scourge mankind has known, the plague, at the time that Mohammed's followers were ready to organize and extend the influence of the crescent until the horns were about to encircle the Mediterranean. Certainly these Arabian tribesmen had never shown any signs of military greatness or valor prior to this period, and it is probable that their religious ardor would have met with small success against the well-organized nations of the time if these nations had not been practically exsanguinated by the "Black Death."

The use of bubonic plague today against a field force, when the forces are actually in contact, is unthinkable for the simple reason that the epidemic could not be controlled. Infected personnel captured would provide the spark to set off possible outbreaks of pneumonic plague in the ranks of the captors. Infected rats would also visit and spread the condition. An advance over terrain infected with plague-bearing rats would be dangerous. Therefore, except as a last desperate, despairing hope of a rapidly retreating army, the use of plague by forces in the field is not to be considered.

The use of plague to harass civil populations presents less difficulty than the use of the organisms against a field force. Those who think that plague will be used as an offensive weapon consider that civil communities may be infected by introducing plague-infected rats. Of course, this is easier to state than to accomplish, but it may be possible for airplanes flying low to drop recently infected rats. At least this is the statement that the individuals make who consider the use of this weapon feasible. Even with so terrible a pandemic disease as plague, however, there is a great deal more to the question of epidemics than mere infection. For instance, to cite an example, one that Gill so forcibly states, "Not half a dozen cases of plague occurred amongst Europeans (including British troops) stationed in the Punjab during the year 1924, when about 500,000, or one-fortieth of the indigenous population suffered from the disease."[1] If these intelligent people were able to avoid the infection when residing in an environment that was literally infiltrated with the infection, it certainly should be possible to control bubonic plague in a population such as we have.

For that matter, the question of plague is not a condition that takes us to the outskirts of civilization. Our own Pacific Seaboard became infected in 1900, and following the San Francisco earthquake the infection extended and is now more or less endemic as a rodent disease, involving not only rats but also ground squirrels. Here again it is not a question of can we control the infection; we are controlling it, and we have not had an outbreak of human plague of sufficient size to designate as an epidemic.

The other insect-transmitted disease that is most frequently assigned a place of importance as an agent suited for warfare is typhus. This disease is certainly terrible enough to satisfy even those individuals who are anxious to preach the gospel of frightfulness. The military and civil populations that have been destroyed by typhus bear witness to how effective this agent of destruction can be. However, again we have a condition that is easily controlled. Complete solution of the problem of endemic typhus is not yet in print, although it is probable that the work of such men as Dyer, Maxcy, and Zinsser will soon offer a complete explanation of how this scourge simmers along during the interepidemic periods. Epidemic typhus is thoroughly understood. The epidemiology is so simple that it can be embraced in the name of the transmitting insect, the body louse. The control of epidemic typhus is the simple question of the control of louse infestations. Of course, quarantine will help to prevent the introduction of the infection, but quarantine is futile if the Army is allowed to become lousy. The lousy Army may become the victim of typhus, even in America, without the introduction of infection from extraneous sources. The weight of opinion in the minds of best epidemiologists is that, as Maxcy suggested, endemic typhus is probably carried over between epidemics in a rodent reservoir. Endemic cases occasionally occur when transmitted to man by an insect, and when the infection is passed from man to man by the body louse, with the resulting enhancement of virulence, epidemics may be expected to result.

The difficulty of starting an epidemic of malaria, yellow fever, or trypanosomiasis (sleeping sickness) appears to be obvious, for no one has suggested the use of these agents. Those who understand the epidemiology of these diseases know they are not suited for war purposes even though they realize the problem they present to military forces in endemic areas.

This completes consideration of the communicable diseases. We have discussed in some detail practically all except the direct contact group. The only diseases of this group of great military importance are venereal, and we have given our reasons for dismissing this group from consideration.

THE INFECTIVE PROCESSES

Certain disease processes that affect the tissues are caused by living organisms and are, therefore, designated as infective, even though they are not considered communicable in the sense that they tend to be transmitted from man to man. These disease processes include such infections as tetanus, gas gangrene, anthrax, and the ordinary pyogenic (pus formers) invaders. The agents that produce these infections have all been mentioned as possible war weapons, and it must be admitted that so far as the first three are concerned, with some scientific judgment on the part of their sponsors.

The agents that cause tetanus, gas gangrene, and anthrax are not delicate organisms such as the relatively short-lived, easily destroyed pathogens that cause most of the communicable diseases. They are very resistant, spore-forming organisms, generally capable of a prolonged period of viability without loss of virulence, even when separated from the animal tissues. It is not surprising, therefore, to find one of this group (anthrax) selected as the infectious agent best suited for military purposes by a science student preparing an undergraduate thesis on "Bacteriologic Warfare."[2] The selection of anthrax does credit to his training. In fact, the entire study shows more intelligent thought than any article that has come to the attention of the writer. His description of the characteristics of the proposed bacterial invader are worth quoting:

> What shall we say are the requirements for a perfect military pathogen? It attacks preferably both man and animals. It must be quick-acting, highly virulent, and capable of causing disease in small quantities. It must be highly resistant, capable of surviving outside the body under the most adverse conditions, and even resisting partial cooking or a careless attempt at sterilization (a spore former). The causative organism should be able to force its entrance through all the avenues of infection; respiratory tract, alimentary tract, and breaks in the skin. The disease should not be too actively contagious, and it must be very well understood, for pathogens should never be used without contemplating the possibility of their getting out of control. Finally, and perhaps most importantly, it should be possible to obtain large quantities of the pathogen in virulent strain and spore form with the least possible manipulation and delay.

After this excellent description of the perfect hypothetical agent, he selects anthrax as the agent best suited to meet the requirements of a bacterial weapon. I cannot agree with Pentler that "Anthrax satisfies the requirements almost perfectly," but I believe all bacteriologists will agree that he has selected the agent that most nearly meets the requirements he has so well outlined.

These spore-forming invaders are a real problem. Tetanus and gas gangrene are pathogenic processes that have always been associated with gunshot wounds and are, therefore, of special interest to the military surgeon. They do not produce epidemic diseases, however, and they are not communicable. They have to have a portal of entry made for them, a wound, and while the use of these organisms to contaminate battlefields might cause an increase in the number of cases of tetanus and gas gangrene, they would not increase the number of casualties. They would only complicate the treatment of those already disabled. It might be added that we have an entirely satisfactory serologic prophylactic agent for tetanus, and that as a result of the surgical advances of the last fifty years, gas gangrene is less frequent than in the prebacteriologic days.

We cannot dismiss anthrax so readily; however, it is worthy of note that although anthrax is almost a world-wide disease, nevertheless anthrax infection of gunshot wounds is practically unknown. If gross contamination of battlefields with the organism of anthrax is effected, it is granted that cases of anthrax infection of wounds will occur, and possibly some few cases of infection in individuals who have not been wounded; but when we consider that human epidemic anthrax is unknown during the bacteriologic era, I question if we need fear greater danger from this organism than contamination of wounds.

It will be noted that up to this point we have not discussed the technical difficulties that a military force would have in contaminating a hostile force. The

difficulties in the case of the communicable diseases are so obvious that they need not be mentioned. The epidemiologic factors make the communicable diseases unsuited for offensive military use. The causative organisms are all either short lived when separated from the living tissues or else readily destroyed by ordinary routine sanitary precautions.

We cannot make this statement concerning the highly resistant infections such as tetanus, gas gangrene, and anthrax. These agents are admittedly the most dangerous; but it must be remembered that to be dangerous they must be alive, and that many technical difficulties present themselves when living agents are to be used that are not present when missiles and chemicals are used. Shells can be used to project missiles and chemicals on to an enemy many miles distant, but bacteria cannot be used in this way. No living organism will withstand the temperature generated by an exploding artillery shell. Airplanes may contaminate terrain, but their effect would be quite local and probably less dangerous and less certain than high explosives used in the same way.

It is not maintained that bacterial contamination is impossible. A retreating enemy may hurriedly contaminate the terrain that is to be evacuated. However, it is believed that the use of living organisms in offensive warfare presents technical difficulties that are not generally considered. The contamination that spies and other individuals could effect, using the only really effective agents we have mentioned—the highly resistant, spore-forming organisms that are so dangerous to wounds—would prove too local to be of any value whatsoever.

TOXIC PRODUCTS

The forms of bacterial warfare include not only the possible distribution of living organisms in the force of, an enemy but also the possible use of toxic products derived from bacteria. Certain of our bacterial toxins are the most deadly poisons known. The toxin of the *Bacillus botulinus* is so powerful that instances have been recorded where toxins have been produced so toxic that 0.005 milligram would kill a 250 gram guinea pig. This material, botulinus toxin, is poison for man. It is possibly the most toxic agent known, and will produce the lethal effect in any way that the material is introduced into the animal. If consumed with food, injected into the tissues, or even dropped on the mucous membran or conjunctiva, it is equally deadly.

This must be the material referred to when we read such dramatic statements as the following: "An airplane can carry sufficient toxins to destroy an entire city." Such statements have an element of truth in them. In fact, they are conservative. An airplane could carry enough of the botulinus toxin to destroy every living man in the world *if* administration of the toxin was as simple a process as production and transportation.

There were over one hundred billion bullets manufactured during the World War—enough to kill the entire world population fifty times; but a few of us are still alive. It is easy to calculate the lethal (fatal) dose of a toxic agent; but do not think it is so easy to figure on the casualty-producing power of a military weapon.

The hostile aviator will not be received with a welcome, nor can he expect to land at an air field near any large city and find the entire population lined up ready to accept the carefully measured lethal dose of botulinus toxin.

The release of tremendous quantities of botulinus toxin over a large city may produce human casualties; however, the extent of the damage might be only the wholesale destruction of rodents, sparrows, and possibly numerous cats and dogs —not such a serious loss in time of war. It is difficult to evaluate properly the possible effects of the bacterial toxins. Certainly such statements as an airplane destroying an entire city with toxins is ridiculous; but they may have a value comparable to chemical agents, with this great disadvantage, however: bacterial toxins are readily destroyed by heat; therefore, like bacteria, they are unsuited for use in shells.

ANIMAL DISEASES

The use of living organisms to produce disease in livestock, such as horses and mules needed for transportation of Army equipment and supplies, has been mentioned as a possible form of bacterial warfare. It is believed that the difficulties here are quite similar to those mentioned for diseases attacking man, with this great advantage to the defense that the veterinary officer will have in controlling epidemics. The veterinary officer can destroy any animal or group that he considers a menace to the health of the animals in the Army. The medical officer cannot take such steps to control epidemics that threaten human populations.

If we expand the term bacterial warfare to embrace such phases of biologic warfare as will include the agricultural pests, then an additional factor to consider is the fact that spies, and possibly hostile aviators, might inoculate growing crops with such pests as the boll weevil, the corn borer, the Mediterranean fruit fly, and like destructive agents. These agents in most instances, however, take so long to invade sufficient terrain to be effective in destroying crops that their value in actually overcoming the resistance of a foe is questionable. They take several years to advance over a large area, and might prove an economic problem years after the war has been completed; therefore, they violate one of the fundamental ideas in warfare, since they would interfere with the ability of the conquered nation to pay the victors for the beating they had received.

CONCLUSIONS

It is believed that it has been shown that the development of implements of warfare represents an evolution based on the gradual application of the improving mind of man. The one factor of importance in this development has been effectiveness. It has been a question of the good mind versus the strong back; of the thinker verses the lifter. It is believed that the future of warfare will be based on the same principles. It is, therefore, apparent that the question of whether chemical munitions will be used or not, and whether bacterial warfare will be used or not, will depend on their practicability rather than on the sentimental reactions of pacifists.

I consider that it is highly questionable if biologic agents are suited for warfare. Certainly at the present time practically insurmountable technical difficulties prevent the use of biologic agents as effective weapons of warfare.

REFERENCES

1. Gill, C. A.: The Genesis of Epidemics, London, 1928. Baillière, Tindall and Cox.
2. Pentler, C. F.: Some Thoughts on Bacteriologic Warfare, Massachusetts Institute of Technology, Department of Biology and Public Health.

CARING FOR THE NAVY'S HEALTH*

CAPTAIN LUCIUS W. JOHNSON, M.C., U. S. NAVY

FEW people had any conception of the vast, intricate organization that made up the Medical Department of the United States Navy before the blooming of the national emergency. Since that time the demands of global war have increased its scope many times, and this growth has occurred with such rapidity that one has more and more difficulty in realizing its size and complexity.

Medical service is provided on shore at recruiting stations, training stations, naval air bases, industrial navy yards, and many other activities. At sea, every ship, large or small, has its medical facilities in accordance with its prospective needs. Numerous bases in our island possessions and on foreign shores, and expeditions sent out to establish new bases, must have adequate combat medical organizations. Procurement and distribution of personnel and material to supply these requirements become a task of great magnitude. It must be remembered that the Navy includes the United States Marine Corps and, for the duration of the war, many activities of the United States Coast Guard.

The policies of the Medical Department are based on the realization that the bluejacket is the essential ingredient in Uncle Sam's struggle to survive. No matter how perfect the guns and the machines may be, the man who operates them is more important. A trained naval man is the result of a long and expensive course of selection and instruction. If one is lost to the service by sickness or injury, it is a serious matter, for his place can be filled only by another who has gone through the same long preparation. Nothing should be denied that will aid in preventing the loss of the services of a single man.

To care for the sick is an important function of the naval doctor, but he has as equal duty to prevent illness and injury. The mission of the Medical Department used to be expressed thus: "To keep as many men at as many guns as many days as possible." Wartime speed-up has now shortened this to: "Keep 'em well." Special effort is made to prepare the doctors entering the naval service for this important phase of their duties. Many have been sent to take special courses in public health work and industrial medicine at some of our leading medical colleges. Others have gone to the Naval Medical School for instruction in naval hygiene, tropical medicine, and other essential subjects.

ACTIVITIES ON SHORE

It is commonly believed that the hazards of the sea and of combat with the enemy are the principal dangers that confront the man of the Navy, but this is

*For reasons that the reader will understand, definite statements concerning the size and location of naval activities have been avoided.

The ideas and opinions here expressed are the private views of the writer and are not to be regarded as the policy of any government department.

All photographs used in this article are reproduced with permission of naval censor.

far from true. The accidents and diseases of the civil community have provided the greatest menace to his welfare. For example, during the last few years motor vehicle accidents on shore have been the greatest cause of death of Navy men, far exceeding drowning. There are many indications that this trend will be changed for the duration of the war.

Transplanting young men from their home towns to new communities for training exposes them to contagious diseases against which they have little immunity. It has been found good practice not to send them aboard naval ships until they have passed a few weeks under medical observation.

The bluejacket's first contact with the doctor is at the recruiting station, where he undergoes his preliminary physical examination. From then on, throughout his naval career, he is under medical surveillance. Forty years ago, Gatewood expressed the opinion that the Navy should keep its very best doctors on duty at the recruiting stations, so that they could detect men having latent defects or diseases, and so exclude them from the service. By doing this, he wrote, the dangers of syphilis and other transmissible diseases could be greatly reduced. The difficulties of accomplishing this are obvious, but it still sounds like an excellent idea. Today, the recruit has his blood tested and an x-ray film taken of his chest, while the psychiatric examination takes on increasing importance.

At the training station a careful medical observation of the recruit is made to detect any hidden defects and to discover contagious diseases in their early stages. Here he receives protective inoculations against a number of diseases, and undergoes a rigorous course of physical training which is designed to uncover any concealed weaknesses. His mental reactions are carefully studied to determine whether or not he can be molded to fit into an organization whose prime need is teamwork.

A concise description of the odd number who cannot work with others is contained in Lafcadio Hearn's statement that the Samurai class in Japan had the right and the duty to behead instantly any inferior person whose conduct was "otherwise than expected." We do not yet dispose of men with schizoid tendencies in this summary manner, but we do try to identify them and determine their potentialities, so that we can employ them where they will be most useful and will do the least harm.

Our industrial navy yards have expanded enormously in size, and many of their departments are working night and day. This imposes a greater mental and physical strain on both shop and office workers. New methods and new materials are constantly being introduced, and they bring with them new industrial hazards which, at first, may be imperfectly understood. It is the duty of the medical officers attached to the yard to study these new developments and to find out what can be done to reduce their menace. At mine and net depots, submarine bases, ammunition depots, aviation training stations, and other naval plants the medical man must be on the alert to detect and alleviate harmful industrial conditions.

Medical establishments are provided at all naval and Marine Corps bases. Those at air bases, navy yards, Marine barracks, and training stations are usu-

ally called dispensaries; it requires special authority from Congress to designate one as a naval hospital. Before our entry into the war several of these dispensaries were complete hospitals with as many as 200 beds. Since then there has been a great increase in their size and number. In staff, equipment, and professional standards, naval dispensaries are equal to hospitals of similar size in civil communities. They provide medical care not only for men of the armed services, but also for civilian employees who are injured or become sick while at work.

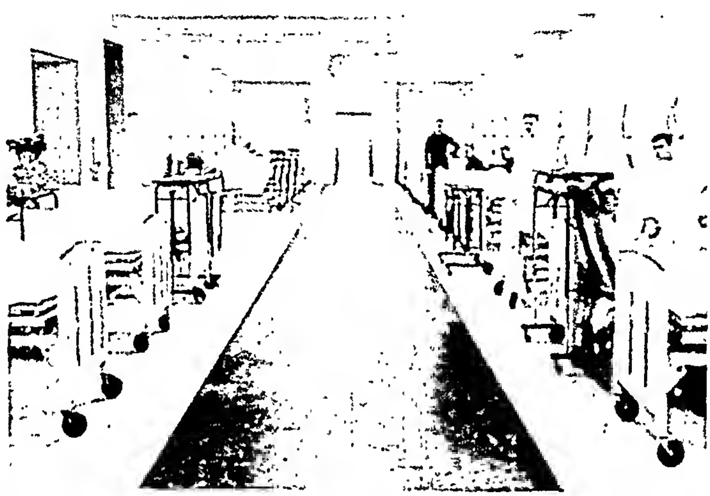


Fig. 1.—One of the wards in a naval hospital. Nursing is done by men of the Hospital Corps, supervised by women of the Navy Nurse Corps.

Naval hospitals are located at strategic points along our coasts, usually near the larger naval bases. Several new ones have been recently built or acquired, and the old ones have been the scene of great activity in the effort to expand them rapidly enough to meet the demands of our augmented Navy. That phase is now nearly completed, and we have time to concentrate on the work of perfecting the equipment and the standards of professional work in our naval hospitals.

Before the present struggle began, hospitals enjoyed an almost complete immunity from hostile attack under the Red Cross flag. In this era of total war they have become special targets, marked for destruction, because loss of hospital facilities has a damaging effect on morale, civil as well as military. The location of most of our naval hospitals, close to large industrial and military establishments, makes them especially vulnerable, and so careful consideration is being given to the need for hospital facilities located farther inland, where the danger of attack would be less. Protection of the existing hospitals is now receiving a great deal of attention. Every necessary step is being taken that will enable them to carry on their work during and after an enemy attack.

ACTIVITIES IN SHIPS AND OVERSEAS

Each of the larger ships of the Navy has its own hospital. Back in the days of Lord Nelson, the increasing weight of armament required that the new

wooden line-of-battle ships be of much heavier construction. This left deep bays on each deck, between the massive timbers. In one of these the hammocks for the sick were slung, and it acquired the name of "sick bay." That term is still used to designate the ship's hospital. A large ship, such as an aircraft carrier, may have as many as 50 to 60 bunks in her sick bay, in addition to an isolation ward, dental clinic, and doctors' offices. Her operating room is as completely equipped as that in a large city hospital. Provision can be made for any necessary operation on any part of the body. On her staff there might be as many as 7 doctors, at least one of whom would be a skilled surgeon. One or more dental officers would form an essential part of the medical staff.

Fig. 2.—A class in chemistry at a Hospital Corps Training School. Many of these men become skilled technicians.

Since there are no accommodations for women on our warships, nursing is done by enlisted men of the Hospital Corps. They are carefully selected from the better-educated recruits, and must have temperaments adapted to the care of sick persons. After their course at the training station is completed, they are sent to Hospital Corps training schools for instruction in their special work. From there they are sent to our larger naval hospitals for a period of practical work in the wards and clinics. Here they are taught by naval doctors and by women of the Navy Nurse Corps. The latter are all graduates of recognized schools of nursing; the Navy does not train any of its nurses.

Many of the men of the Hospital Corps become highly-skilled technicians in the various medical specialties. An index of the success of their course of training is found in the constant demand for navy-trained men when their enlistments expire. Large industrial organizations desire them for male nurses, first-aid

men, and technicians in laboratory, x-ray, or other special work. Throughout the Navy they provide a very satisfactory nursing and technical service.

Smaller ships, such as destroyers, have a man of the Hospital Corps attached to each, while the mother ship of the group has a large sick bay with medical and dental staff, and complete clinical facilities. It is the purpose of the Medical Department to bring its services close to every man of the Navy and Marine Corps, no matter how distant he may be, or how small the unit to which he is attached. One of the important factors in maintaining the morale of small organizations in remote locations is the assurance that a sick or injured man will have adequate medical attendance.

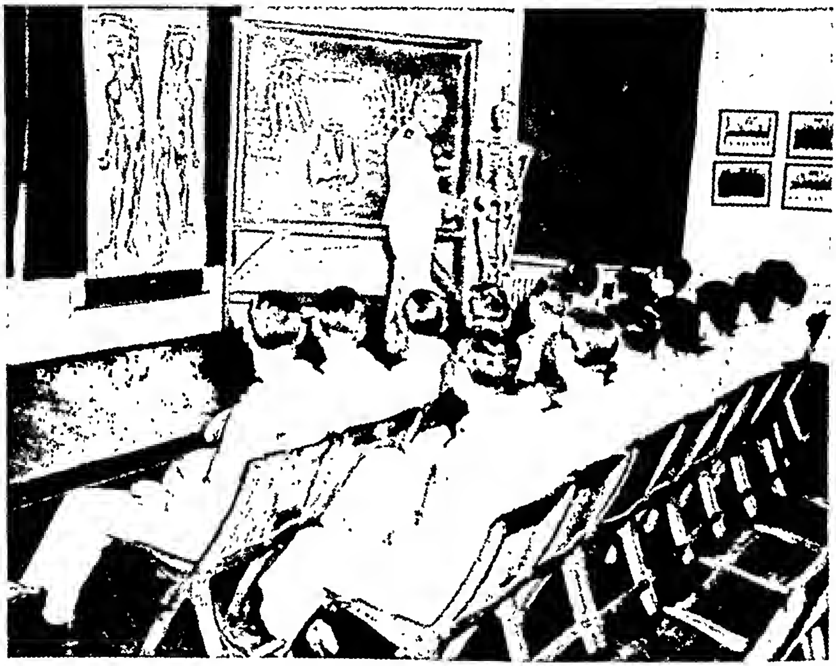


Fig. 3.—Class in anatomy at a Hospital Corps Training School.

Expeditionary forces are an important feature of the Navy's work. The Marine Corps is a unique, amphibious outfit, designed to act as a spearhead that can be advanced with great rapidity over long distances, to seize and hold advanced bases. This highly specialized duty requires the medical organization and equipment to be carefully planned, so that the doctor may be there with his necessary materials at the critical moment. To many medical officers this is the most interesting phase of the Navy's work. Many ingenious plans and gadgets have been devised by various medical officers during their study of the problems of expeditionary duty.

Many new and intricate problems of public health are imposed by the Navy's recent occupation of bases on foreign shores. At several of these, the prevalent diseases of the local people may be a greater danger than any action of the enemy. Problems of food, clothing, and living conditions must be studied, and unsanitary conditions must be corrected before the fighting forces can maintain their highest combat efficiency.

Our Navy is undergoing a precipitate change in character as a result of the lessons of recent months, with the air force becoming the dominant branch. This places on the Medical Department the obligation to develop ways and means of providing medical care under the changing conditions. New hazards and new difficulties are being encountered almost daily, so that an enormous amount of research is required to define the new problems and to find solutions for them. Many medical officers are now under training in the important specialty of aviation medicine, and the aid of some of the ablest scientists in the country has been obtained for research in the medical problems of flight.

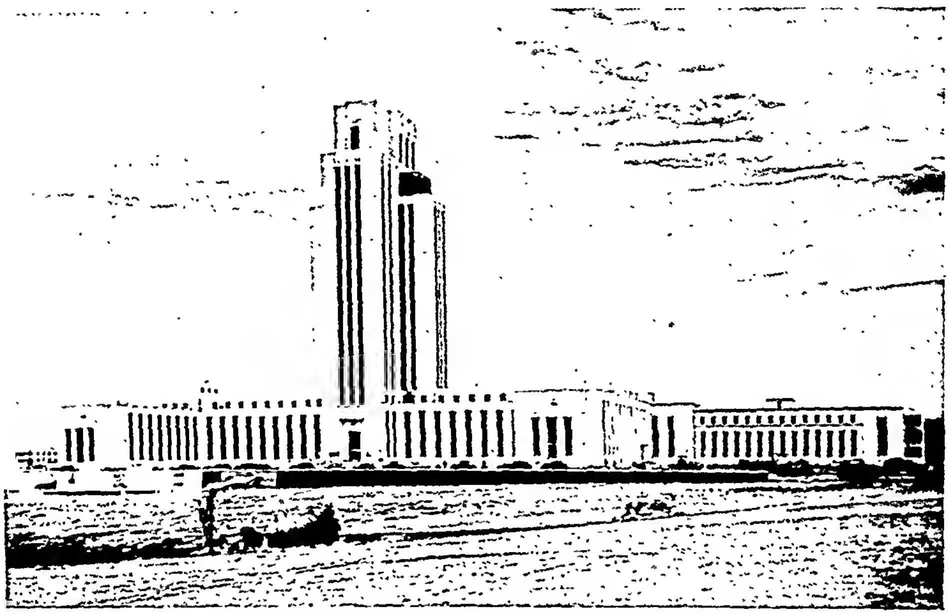


Fig. 4.—The new buildings of the Naval Medical Center, at Bethesda, Maryland, were recently dedicated by President Roosevelt.

NAVAL MEDICAL INSTITUTIONS

First among these is the Naval Medical Center, located in Bethesda, Maryland, a suburb of Washington. The magnificent new buildings which house its many activities have recently been dedicated to their beneficent purpose. The group includes the Naval Hospital, Naval Medical School, Naval Dental School, the research activities, and schools for training technicians in many special branches. The buildings were designed and equipped to equal the facilities of the best hospitals and professional schools throughout the country. Since all medical and dental officers enter the Navy after they have completed their professional education, these schools offer postgraduate courses.

Before the rapid expansion of the Navy began, it was the policy to send newly commissioned medical and dental officers to these schools to learn the ways of the service, and also to receive instruction in several specialities which are of little interest to the general practitioner, but are of vital importance to the naval doctor. These included naval hygiene, tropical medicine, emergency surgery, public health work, and an outline of the problems of diving, aviation, and submarine work.

Development of naval hospitals has been rapid since we entered the war. New ones have been taken over, and the old ones have been greatly enlarged. They are staffed and equipped so that the patients may have everything and every care that may be helpful in their recovery. It is the aim of Admiral McIntire, our Surgeon General, to have the equipment and the professional services in naval hospitals equal to the best that can be found anywhere in the country. Nothing is spared that will aid in accomplishing this purpose.

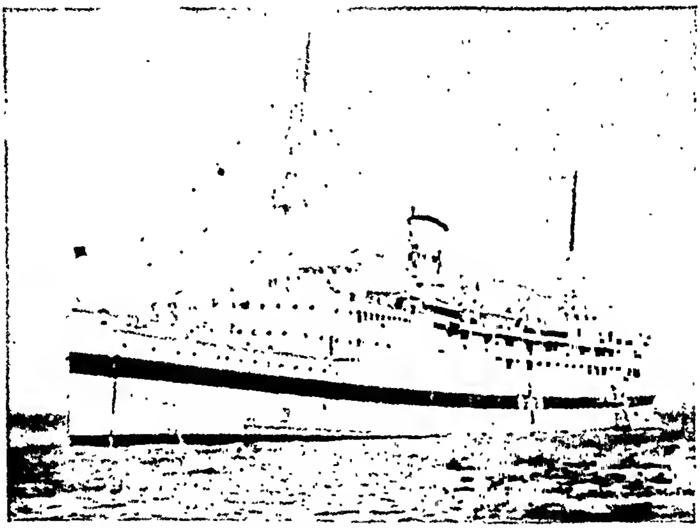


Fig. 5.—U. S. S. Solace, one of the hospital ships that accompany the fleet. She was commissioned in August, 1941.

Floating hospitals perform an important function in our Navy. If you can imagine a force of 50 or more ships, with 45,000 men, shoving off from its base for prolonged operations in distant waters, you will realize that it requires hospital facilities at least equal to those of an industrial city of that many people. Hospital ships have the staffs and equipment that will provide complete service in all the clinical specialties, and they are also prepared to act as public health laboratories. They serve the fleets at their bases, and they are able to accompany them when they proceed on their distant missions.

Much attention has recently been devoted to mobile hospitals, and they have proved their value in several combat areas. They are hospitals of several hundred beds, designed, staffed, and equipped so that they may be quickly moved by land, sea, or air, and rapidly set up when the new location is reached. Their purpose is to serve advanced bases, and to furnish hospital facilities as soon as possible after an expedition to a remote area has landed. If required, they can send off smaller units to serve subsidiary bases. An infinite variety of methods can be employed to adapt them to the needs of special areas, or to different types of military organizations.

MEDICAL DEPARTMENT PERSONNEL

For many years the Navy obtained its medical officers by competitive examination of men from class A medical schools. More recently, selected graduates

were admitted for intern training in naval hospitals. Since the emergency dawned, reserve medical officers have been called to active duty so rapidly that they now greatly outnumber those of the regular Navy. The medical officers of the Naval Reserve were carefully selected men, outstanding members of the profession in communities all over the country. When the call to duty came, they gave up their practices, severed their profitable civilian connections, and devoted their talents to serving the needs of Navy men. This influx of new men with new ideas and new talents has been of great value to the service, for many were teachers and research workers of unusual ability. It can be safely stated that nowhere in the country is there better professional service than that provided for the bluejacket in the average naval hospital.

Fig. 6.—Medical ward in U. S. S. Relief, hospital ship, which was commissioned in 1920.

In our naval hospitals the medical officers carry a gradually increasing load of administrative responsibility throughout their careers. The young medical officer in charge of a ward is responsible for the cleanliness and good discipline of his area, as well as for the professional care of his patients. This helps to develop rivalry and a sense of pride in the appearance and functioning of the whole institution. By the time a man becomes executive or commanding officer of a hospital, he is thoroughly familiar with most of the details of the uniform system of administration. The administrative system is constantly kept up to date by adopting the best methods observed in civil hospitals.

Dentists are appreciated as very valuable members of the Medical Department team. In the old days of the Navy, if a man had enough teeth to pass the physical examination for enlistment, he received no further official attention. It is different now. Every effort is made to put the teeth of every man in first-class condition during his period of training, and to maintain them in that con-

dition throughout his time in the service. An infinite amount of suffering is thus prevented.

The dentist in the Navy performs many other important functions. He is usually called upon to collaborate in the treatment of fractures about the face and jaws, and frequently assists in operations in this field. His special training in regional anesthesia enables him to give the general surgeon much-needed aid in many cases. During the pioneer work with Naval Mobile Base Hospital No. 1, the dental officer was of invaluable assistance and performed with enthusiasm many duties far outside his professional field. At Pearl Harbor and other combat areas, the first-aid work done by the dental officers has received the highest praise. Reserve dental officers, like their medical colleagues, have reported for active duty in great numbers, and are gladly welcomed as an important addition to the staffs of naval activities.

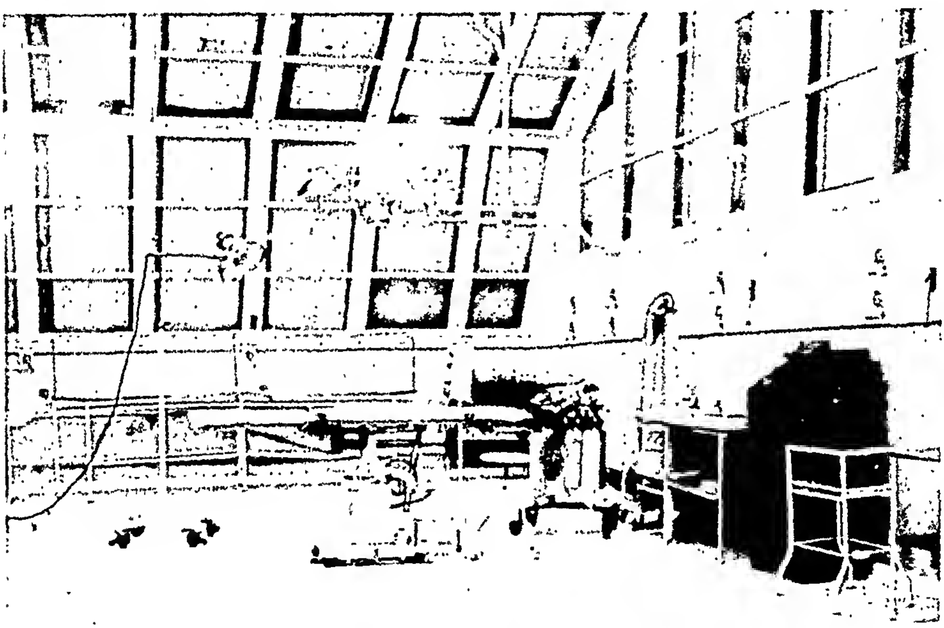


Fig. 7.—Port side of the operating room, U. S. S. Relief. Operations can be done while at sea, just the same as in port.

The Navy Nurse Corps is composed of female graduates of recognized nursing schools, who undergo careful scrutiny and selection before being appointed. The reserve nurses were organized with the aid of the American Red Cross. In our naval hospitals these women maintain a very high standard of nursing service. They also perform a very valuable function in training the men of the Hospital Corps. The obligation of our naval hospitals to provide well-trained corpsmen for duty on ships and at remote bases is second in importance only to the care of patients. A bill has recently been passed and approved which gives to nurses in the Navy increased pay and a definite status as officers. This is a well-merited recognition of their valuable service to the Navy.

Pharmacists are men who have served for many years in the Hospital Corps. They have long been restricted to warrant grades, but recently authority has

been given for their advancement to commissioned ranks. Their duties have little to do with pharmacy, but are more concerned with property and accounting, commissary, and personnel work. They are invaluable administrative aids, and no commanding officer could wish for a greater blessing than to have two or three old-time naval pharmacists on his staff.

Men of the Hospital Corps have frequently been spoken of as the most versatile men to be found anywhere, and there is ample justification for this. They are trained in nursing and the technical specialties of the medical field. Service with the Marines teaches them the use of tools and weapons, and develops their ability to take care of themselves in the field under all sorts of conditions. Duty on board ship gives them some knowledge of many mechanical trades, as well as an understanding of nautical life. Clerical work and accounting are also a part of their training. The result of all this is an alert, well-disciplined man, who is fitted to make his way in life, and to be a useful member of any organization.

An outline has been presented of the means by which the Medical Department of the Navy acts to perserve the health of its men and to care for those who are sick or injured. A severe test of the efficiency of the organization was provided by the surprise attack at Pearl Harbor, December, 1941. It is gratifying to be able to state that the preparations were found to be adequate. The Naval Hospital, the hospital ship, and Naval Mobile Base Hospital No. 2 were able to care for all the naval casualties without having to call for outside aid. Disinterested observers, who later examined the injured, spoke with praise of their excellent condition. They stated that never before had a large number of seriously-injured persons made such favorable progress.

If the general public, and especially the relatives and sweethearts of men in the Navy, could know what complete and elaborate preparations are made for conserving their health, it would be a great solace to them. Uncle Sam neglects nothing that will aid in preserving and restoring the health of the men of his Navy.

THE DEFINITION AND SCOPE OF NAUTICAL MEDICINE

CAPTAIN LOUIS H. RODDIS, M.C., U. S. NAVY

MOST civilian medical men realize that nautical medicine is a specialty which is as definite and distinct as the other professional medical specialties and that a specialist in naval medicine occupies a particular field as much as does a specialist in pediatrics or surgery.

The terms "nautical," "maritime," and "naval medicine" are three terms which are often considered as synomons. This is perhaps true of nautical and maritime medicine. Naval medicine, however, has a somewhat different meaning. Nautical or maritime medicine may be defined as that branch of medical science which has to do with the prevention and treatment of diseases and injuries peculiar to maritime or nautical life. Naval medicine may be defined as that branch of nautical medicine which is particularly concerned with diseases and injuries incident to both life at sea and naval warfare.

One of the subjects of special importance to the seagoing doctor is that of naval hygiene, which includes problems connected with ventilation; air conditioning; heating; waste disposal on board surface ships, submarines, and closed cabin aircraft. Other subjects of study are physical changes in relation to rapid changes in climate, an important matter in maritime medicine, where a man may be in a polar latitude one week and the temperate zone or tropics a short time later; the question of water supply at sea; production of fresh water from sea water or from the moisture of the air; bacteriology of the air at sea and the bacteriology of sea water; the care of the sick and injured aboard ship; the structural feature of the ships in relation to health; the control of disease vectors aboard ship; the study of epidemiology as affected by living conditions aboard ship and the prevention of diseases under conditions of shipboard life; the study of such a particularly nautical disease as seasickness and injuries from special nautical hazards, such as falls from masts and down hatches, injuries from handling boats, anchors, and similar accidents of nautical life; wounds inflicted by fish and marine animals; inspection and preservation of food for ocean voyages; proper rations, including special emergency rations for planes, boats, and submarines; and the transportation of the sick and wounded aboard ship, from ship to ship, and from ship to shore.

To such problems as these are added additional ones which may be considered as peculiar to navies and pertaining to naval medicine. Such are the physical standards of physical examinations for entrance into the naval service; the caring for wounded in battle and after battle; the disposition of battle dead; the employment of ambulances and hospital ships and of hospital and ambulance planes; the study of first-aid materials for the individual soldier and sailor; the tactical employment of medical troops with expeditionary forces and landing parties; the treatment of gas casualties; postgraduate training of med-

ical officers and the training of hospital corpsmen and technicians; the maintenance of naval hospitals and naval medical supply depots; the obtaining of drugs and medical and surgical supplies and equipment for use on ships and at naval hospitals; the disposition of cases with permanent disabilities and pensions; the caring for the tubercular and the insane of the Navy; the questions of naval medical history and of naval medical organization and administration; the uniforms and insignia of the medical department of navies; the practice of pharmacy and dentistry in navies and of the utilization of female nurses in naval establishments. All these represent problems and fields in naval medicine, and the naval medical officer must be cognizant of them and know something about them. Training and experience are needed to obtain this knowledge just as in other specialist branches of medicine.

The naval medical officer must be an excellent general practitioner. Often he is in isolated places or the only medical men on a ship in the middle of a large ocean. The medical problems of the men and officers of that ship must be solved by him and him alone. He cannot turn to a specialist or refer a case to a surgeon, an eye, ear, nose, and throat specialist, or a dermatologist just across the street. He must deal with the case himself. I do not mean by this that specialism is not needed in the Navy. As a matter of fact, every specialty must be represented and in the hospitals, and places where definitive treatment can be given specialists in every specialty must be available. I wish, however, to emphasize the point that the naval surgeon must be a good general practitioner first of all, with some definite knowledge of naval medicine. It is said that the specialty of the general practitioner in civil life is obstetrics. I would say that the specialty of the naval surgeon is naval medicine and that both preventive medicine and traumatic surgery form essential features of his specialty.

The versatility of the naval surgeon and the men of ability which his specialty has attracted is shown in the history of nautical medicine. Many of the greatest names in medicine and science have come from the ranks of naval surgeons: James Lind, Sir Gilbert Blane, Linnaeus, Darwin, and Huxley were naval surgeons. The founders of tropical medicine were naval surgeons. Even the surgeon of a crew of buccaneers is known to every medical man today who prescribes Dover's powder. The history of medicine, indeed, owes much to the seagoing medical man.

ADAPTATION OF TECHNICIAN INSTRUCTION TO THE MILITARY EMERGENCY*

MAJOR JACK W. LOVE, M.C., CAPTAIN I. ROTHSTEIN, M.C., AND FIRST LIEUT. J. CUBEL, M.A.C.

CONSIDERABLE interest has been shown recently in the type of training given to enlisted men in the United States Army to equip them to work as laboratory technicians. This interest, apparently, has been stimulated by the realization that we have only a period of twelve weeks in which to prepare these students to assume the many responsibilities of a laboratory technician. It is our purpose to outline the working schedule of training, and we trust that inspection of it will indicate the scope, objective, and methods of instruction used in this course.

The Laboratory Section of the School for Medical Department Technicians has a teaching staff of three full-time officer instructors, one staff sergeant, one sergeant, and three enlisted specialists who act as assistant instructors. As part-time assistant instructors there are other more technically trained specialists who contribute their services during certain periods of the course. The technical background of the staff has been largely acquired in the field of clinical pathology, including the various specialties.

As the training schedule is unfolded, one will readily see that in order to cover the various subjects within the three-month period allowed for this course, there must be a sacrifice of either the formal didactic lectures or the practical "bench work" training. Consequently, in order to have the student derive the utmost from the course, it is so arranged that there is a minimum of lectures; these consist of outlining the particular work to be covered for that day, stressing the salient features and placing emphasis on the more important aspects involved in the performance of the test. The student's interest and proficiency are maintained at a high level by occasionally introducing the clinical application and interpretation of the routine procedure.

In order to fit this course into a period of twelve weeks, the hours of instruction are from 8:00 A.M. to 4:00 P.M., with an hour for lunch at noon. Saturday afternoons and Sundays are given as rest periods, and extra time in the laboratory at night is discouraged except for those students who cannot keep up this rapid pace. This results in a total of 460 hours of training, with the allotment as follows:

*From the Laboratory Section of the School for Medical Department Technicians, Army Medical School, Washington, D. C.

a. Basic Technique	57 hours
b. Hematology	60 hours
c. Parasitology	57 hours
d. Bacteriology	108 hours
e. Histologic Technique	48 hours
f. Chemistry	90 hours
g. Serology	36 hours
h. Advanced Hematology	4 hours
	460 hours

Tables I-III show the daily program divided into three four-week periods following in direct sequence.

Basic Technique.—As this subject implies, each student is instructed in the simpler routine laboratory procedures which are essential in the maintenance and care of supplies and equipment used in clinical pathology. The student is taught to clean glassware properly and to acquaint himself with the various methods of sterilization. He is taught the preparation of stains and solutions, the simpler technique of inoculating solid and liquid media, the transferring of cultures, staining of bacteria, and the making of the so-called "wet" preparations for study. Here the practical "bench work" method of instruction consists of allowing each student to perform this work under proper and adequate supervision.

Hematology.—The object in this part of the course is to train the student to perform a simple blood count with accuracy. The expression "simple blood count" includes the hemoglobin determination, the erythrocyte and leucocyte counts, and the differential white cell tabulation. In regard to other hematological procedures, such as the platelet count, reticulocyte count, peroxidase differential stain, bleeding and coagulation times, only a minimum amount of time and instruction are given in order to give the student merely an insight into these procedures so that each can be performed under supervision. In the second four-week period, four hours of instruction are given in advanced hematology. This time is devoted to demonstrations of the various blood disorders in conjunction with enough didactic instruction to orient the student with regard to the important blood disorders and to help him realize the necessity of assistance from the laboratory director when abnormal cells are encountered during the performance of a simple blood count.

Serology.—The beginning part of this subject is devoted to both demonstration and practical application of venipuncture technique, of the separation of sera from blood specimens, and of setting up both blood typing and crossmatching tests along with their interpretation. The more detailed serologic procedures consist of repeatedly performing the standard and quantitative Kahn tests for the diagnosis of syphilis. In addition, the student is taught to prepare colloidal gold solution and to perform several colloidal gold tests on spinal fluids. The Kolmer complement fixation test for syphilis is also demonstrated.

Histologic Technique.—In this course each student is taught the proper care of the equipment used in the preparation of tissue for histologic examination; this includes the care and sharpening of the microtome knife. Instruction is given in tissue fixation and embedding, and sectioning of tissue with both the paraffin and frozen section methods. In the more advanced part of this train-

TABLE I

FIRST FOUR-WEEK PERIOD

LABORATORY TECHNICIAN TRAINING—S.M.D.T.

8-12 A.M.	*1st Day* Hematology Hemoglobin determination Technique of finger puncture Practical—application of above	*2nd Day* Hematology White blood cells Blood smears, staining, differential count Practical—application of above	*3rd Day* Hematology Errors in differential counts Errors in hemoglobin determination Practical—differentials	*4th Day* Hematology Blood cell counts Calculation of red and White cells Practical—complete blood count	*5th Day* Hematology Errors in red and white cell counts Practical—two complete blood counts	*6th Day* Examination in Basic Training.
1-4 P.M.	Basic Training Outline of course Laboratory rules Care of equipment	Basic Training Cleaning glassware Cleaning—Pipettes, syringes needles, etc.	Basic Training Plugging glassware Wrapping glassware Cleaning new glass	Basic Training Use of thermometer Fahrenheit scale Centigrade scale	Basic Training Glass bending Glass cutting Capillary pipettes·	
8-12 A.M.	*7th Day* Hematology Review—blood counts and calculations Practical—two complete blood counts—reports	*8th Day* Hematology Blood platelets determination importance Practical—blood platelet determination	*9th Day* Hematology Errors in platelet determination Practical—blood count, differential, platelet determination	*10th Day* Hematology Reticulocyte Determination, supra vital staining Practical—reticulocyte Determination, calculation reports	*11th Day* Hematology Errors in reticulocyte counts—review Practical examination on complete blood counts	*12th Day* Examination in Basic Training
1-4 P.M.	Basic Training Sterilization of tongue depressors, throat swabs	Basic Training Use and care of laboratory animals Inoculation of above	Basic Training Elementary chemistry Symbols, elements, compounds Use of balance	Basic Training Solutions—saturated, dilute and normal preparation of solutions	Basic Training Culture media—preparation, adjustment of reaction, storage	

8-12 A.M.	*13th Day* Hematology Errors in examinations Abnormal red cells Practical—blood counts with cautions against errors made in examination	*14th Day* Hematology Peroxidase reaction Use and performance Method of reporting Practical—application of peroxidase reaction	*15th Day* Hematology Coagulation time Bleeding time Practical—application of above	*16th Day* Hematology Review of high points of complete course Practical—review of blood study procedures	*17th Day* Hematology Final examination Practical—W.B.C., R.B.C., color index, hemoglobin determination, and differential	*18th Day* Examination in Basic Training
1-4 P.M.	Basic Training Preparation of media, infusion broth, extract agar, E.M.B. plates	Basic Training Prepare blood agar slants and blood agar plates	Basic Training Demonstration of motile and non-motile bacteria Hanging drop and wet preparations	Basic Training Staining—Gram's stain Loeffler's stain Preparation of dyes for stains	Basic Training Practice in staining Bacteria using Gram's stain Bacteria of sputum, feces	
8-12 A.M.	*19th Day* Chemistry Urine collection constituents of urine Practical—physical and chemical examination	*20th Day* Chemistry Organized and unorganized sediments in urine Practical—microscopic examination of urine	*21st Day* Chemistry Importance of routine urinalysis Practical—complete urinalysis	*22nd Day* Helminthology Classification of worms Life cycles and habitat Practical—examination of unknown feces for eggs	*23rd Day* Helminthology Wet preparations Practical—examination of unknown feces	*24th Day* Final examination in Basic Training Helminthology continued
1-4 P.M.	Basic Training Inoculation of Media Pure cultures supplied	Basic Training Isolation of pure cultures of bacteria from mixed cultures Morphological and cultural characteristics	Basic Training Collecting of specimens and preparation for shipment Practical—application of above	Serology Venipuncture Use of necessary instruments Practical—each man does a venipuncture	Serology Blood typing—importance, method, classification Practical—application of above	

TABLE II

SECOND FOUR-WEEK PERIOD

LABORATORY TECHNICIAN TRAINING—S.M.D.T.

8-9 A.M.	*1st Day* Histology General introduction Fixing fresh animal tissue	*2nd Day* Histology Tissue fixing Introduction to staining eosin, etc.	*3rd Day* Histology Further work with eosin and hematoxylin	*4th Day* Histology Paraffin Embedding, further fixing and staining	*5th Day* Histology Paraffin Embedding and further staining	*6th Day* Histology Paraffin embedding and further staining
9-12 A.M.	Helminthology Review—charts and slides, etc. Practical—examine three unknown feces	Parasitology Characteristics of amebae, importance Practical—*E. coli*, *E. histolytica*	Parasitology Examine feces with cysts Examine cultures with trophozoites	Parasitology Examination—feces containing helminth eggs and amebic cysts	Parasitology Blood and tissue flagellates—*giardia Trichomonas*, *Leishmania*	Parasitology Malaria—life cycle, importance, *P. virax* Practical—examine slides
1-4 P.M.	Serology Cross matching Blood transfusion Practical—blood typing, cross matching	Serology Serological tests Care of glassware Practical—wash glassware	Serology Kahn test, antigen reagents, technique Practical—run a Kahn test	Serology Examination—venipuncture, blood typing, cross matching	Serology Serological forms Shipment of specimens Practical—each man will run 6 Kahn tests	
8-9 A.M.	*7th Day* Histology Further embedding and staining Introduction to decalcification	*8th Day* Histology Embedding, staining, and decalcification	*9th Day* Histology Paraffin embedding, decalcification Study of animal tissues	*10th Day* Histology Introduction to celloidin method Further work in decalcification	*11th Day* Histology Further work in study of animal tissues Further work in the celloidin method	*12th Day* Histology Further work in celloidin embedding, tissue study, paraffin embedding
9-12 A.M.	Parasitology Malaria—compare *P. Vivax* with *P. Malariae* Practical—examine slides of *P. Malariae*	Parasitology Malaria—*P. falciparum*, importance Practical—examine slides of *P. falciparum*	Parasitology Review—all types of malaria with unknown slides Demonstration set up	Parasitology Entomology—roles played by insects, mosquito Practical—study anatomy of mosquito	Parasitology Conclude study of mosquitoes started the previous day	Parasitology Fleas, ticks, lice, bedbugs, etc., medical importance Practical—study set up exhibit

1-4 P.M.	Serology Spinal fluid Kahn Quantitative Qualitative Practical—run a spinal fluid Kahn test	Serology Colloidal gold preparation Practical—each man will run two colloidal gold tests on spinal fluid	Serology Read test set up previous day—report Demonstrations in Serology Department	Serology Review complement fixation test Practical—each man will read 15 colloidal gold tests	Serology Final written examination in serology	
8-9 A.M.	*13th Day* Histology Further work in celloidin embedding Cutting paraffin sections	*14th Day* Histology Celloidin method Cutting of paraffin sections	*15th Day* Histology Cutting of paraffin sections and further tissue study	*16th Day* Histology Introduction to cutting and staining frozen sections	*17th Day* Histology Cutting and staining frozen sections	*18th Day* Histology Cutting and staining frozen sections
9-12 A.M.	Parasitology Study various types of flies—their medical importance Practical—study fly exhibit	Parasitology Review of entire course Practical—examine unknown stool and blood specimens	Parasitology Final examination covering unknown stools, slides, and demonstration	Bacteriology Classification of bacteria—*Staphylococcus albus* and *aureus* Practical—streak blood plates, make Gram stain	Bacteriology Examine plates made previous day, cultural characteristics Streak blood plates with *Staph. pyogenes* and *Staph. salivarius*	Bacteriology Examine plates made previous day Characteristic Gram's stain of organisms
1-4 P.M.	Hematology Anemias of all types Polycythemia Purpuric—hemorrhagic diseases	Hematology Leucemia—acute and chronic types Bone marrow studies, etc.	Chemistry Quantitative examination of urine	Chemistry Quantitative examination of urine	Chemistry Quantitative examination of urine	
8-9 A.M.	*19th Day* Histology Introduction to Giemsa's staining for bacteria	*20th Day* Histology Further work with Giemsa's stain for bacteria	*21st Day* Histology Cutting and staining celloidin sections	*22nd Day* Histology Cutting and staining celloidin sections	*23rd Day* Histology Practical examination Identification of tissues	*24th Day* Histology Written examination on technique procedures
9-12 A.M.	Bacteriolgy Technique of blood cultures Practical—make a blood culture	Bacteriology *D. Pneumoniae* Identification Inoculation of plates Neufeld reaction	Bacteriology Study *D. pneumoniae* Bile solubility test Examine blood plates for type of colony	Bacteriology Genus *Neisseria* Inoculation, Gram's stain, characteristics Diagnosis	Bacteriology Examine plates of Neisseria organisms Examine smears from acute cases	Bacteriology *N. intracellularis* characteristics Polyvalent and type agglutination test
1-4 P.M.	Chemistry Quantitative Examination of urine Examination	Chemistry Gastric analysis introduction Physiology, motion picture	Chemistry Gastric analysis Titration of gastric contents	Chemistry Gastric analysis Titration of gastric contents	Chemistry Gastric analysis examination—titration of unknown gastric cont...	

TABLE III

THIRD FOUR-WEEK PERIOD

LABORATORY TECHNICIAN TRAINING—S.M.D.T.

8-9 A.M.	*1st Day* Histopathology Bacterial staining Giemsa stain	*2nd Day* Histopathology Bacterial staining Giemsa stain	*3rd Day* Histopathology Bacterial staining Giemsa stain	*4th Day* Histopathology Bacterial staining MacCallum's stain	*5th Day* Histopathology Bacterial staining MacCallum's stain	*6th Day* Histopathology Bacterial staining MacCallum's stain
9-12 A.M.	Bacteriology Read agglutination tests previously set up Examine G. C. slides Examination	Bacteriology Water examination technique, importance Practical—run a water examination	Bacteriology Continuation of water analysis and counting of colonies	Bacteriology Autogenous vaccine and its application Continue water analysis	Bacteriology Continue water analysis—milk analysis—test on vaccine	Bacteriology Milk count and complete water analysis Make final report on vaccine sterility test
1-4 P.M.	Blood Chemistry Preparation of chemicals used in blood chemistry	Blood Chemistry Completion of solutions, make up Nessler's reagent	Blood Chemistry Calorimetry—explained and applied	Blood Chemistry Blood filtrate preparation Sugar determination	Blood Chemistry Nonprotein nitrogen determination	
8-9 A.M.	*7th Day* Histopathology Fat staining Sudan iv	*8th Day* Histopathology Fat staining Sudan iv	*9th Day* Histopathology Fat staining Sudan iv	*10th Day* Histopathology Fat staining Sudan iv	*11th Day* Histopathology Tubercle bacillus staining Mallory's method	*12th Day* Histopathology Tubercle bacillus staining Mallory's method
9-12 A.M.	Bacteriology Gram-negative enteric bacilli Inoculation of *E. typhosa*, para A and para B	Bacteriology Identification of pathogenic enteric bacilli—procedure	Bacteriology Further work with enteric bacilli inoculation cultures	Bacteriology Further work with enteric bacilli Examine cultures	Bacteriology Further work with enteric bacilli Agglutination tests	Bacteriology Food poisoning Read agglutination tests previously set up

1-4 P.M.	Blood Chemistry Urea nitrogen, uric acid, creatinine determinations	Blood Chemistry Urea clearance Collection of specimens	Blood Chemistry Urea clearance determination	Blood Chemistry Glucose tolerance test and determination	Blood Chemistry Sulfonamide chemistry Sulfanilamide determination	
8-9 A.M.	*13th Day* Histopathology Iron hematoxylin stain for fibrous tissue	*14th Day* Histopathology Iron hematoxylin Stain for fibrous tissue	*15th Day* Histopathology Weigert's stain for elastic tissues	*16th Day* Histopathology Mallory's phosphotungstic stain for fibrous tissue	*17th Day* Histopathology Mallory's phosphotungstic stain for fibrous tissue	*18th Day* Histopathology Mallory's phosphotungstic stain for fibrous tissue
9-12 A.M.	Bacteriology Prepare antigens Set up agglutination tests made with above antigen Examination	Bacteriology *Vibrio comma* Inoculation of non-pathogenic *V. Comma*	Bacteriology Further work with *V. Comma*—genus Brucella	Bacteriology Spore-forming bacteria genus *bacillus* Aerobic culture methods	Bacteriology Spore-forming bacteria genus *Clostridium* motility tests	Bacteriology Spirochetes—*Treponema*, *Leptospira* and *C. borrelia* (Vincent's angina)
1-4 P.M.	Blood Chemistry Calcium and phosphorus determinations	Blood Chemistry Liver function test	Blood Chemistry Carbon dioxide and carbon monoxide determination	Blood Chemistry Phosphatase determination	Blood Chemistry Cholesterol determination	
8-9 A.M.	*19th Day* Histopathology Stains for hemosiderin	*20th Day* Histopathology Stains for hemosiderin	*21st Day* Histopathology Silver impregnation for syphilis	*22nd Day* Histopathology Final examination	*23rd Day* Histopathology Clean up the laboratory	*24th Day* Graduation Exercises
9-12 A.M.	Bacteriology *Corynebacterium* *C. diphtheriae* *C. xerosis*, etc.	Bacteriology Virulence test for *C. diphtheriae*	Bacteriology Mycobacterium Ziehl-Neelsen staining	Bacteriology *Mycobacterium* concentration of specimens	Bacteriology Yeasts and molds Final examination	
1-4 P.M.	Blood chemistry Serum protein determination	Blood Chemistry Clean apparatus for the final examination	Blood Chemistry Final examination Practical	Blood Chemistry Clean up laboratory Prepare solutions	Toxicology Lecture with demonstrations	

ing, the student is taught to use various stains, with their respective indications and value, although emphasis is placed on the routine hematoxylin eosin and Masson trichrome stain techniques. The hematoxylin eosin stain is used on tissues that are carried through the entire routine by each student. Naturally, in teaching tissue selectivity, a few essentials of histology are included so that the student may, to some extent, recognize the different tissues under the microscope.

Parasitology.—Throughout this entire course practical "bench work" is stressed, and the only lectures given are to guide the student in his routine work and to point out the more important features used in the identification of the various parasites. The course starts with the study of the various helminth ova, using both fixed preparations and stool specimens; then gradually the more detailed studies are added, which include identification of the malarial parasites, insects, and arthropods. Each student is equipped with a loan set of slides of fixed preparations, and considerable time is devoted to the study and practical examination of these slides.

Bacteriology.—In this course each student is familiarized with the practical aspects of bacteriologic technique, especially that part which will enable him to isolate and identify tentatively the various bacteria of medical importance. During the six weeks of three-hour periods, each of the genera is studied, with emphasis being placed on the more important species. In dealing with the more common pathogenic bacteria, a morphologic and cultural study is performed, and where indicated, differential media, biochemical, serologic and animal tests are used. The special procedures covered during this course include the performance of blood cultures, pneumococcus and meningococcus typing, water and milk analysis, agglutination tests, anaerobic culture methods, and the dark-field technique.

Chemistry.—The student is first taught the simple and practical fundamentals of chemistry. With this background, the course embraces the various phases of a routine urinalysis, including the physical, chemical, and microscopic examination, using normal and abnormal urine samples. Practical instruction is then given in titration, normal solution preparation and both are utilized in the performance of a fractional gastric analysis. The final four-week schedule has each afternoon devoted to metabolic chemistry. A large part of this scheduled time is used in the actual performance of the various metabolic determinations requested of a well-organized chemical section of a medical laboratory. In this more detailed portion of the course, it is only natural to include some of the practical working theories, but these are only dealt with from their useful and practical aspects.

Tables IV, V, VI, and VII represent graphically an analysis of twelve successive classes of students trained during the first year of this course (Laboratory Section, S.M.D.T.) of the Army Medical School. Table IV reveals that half of the students admitted were college students, college graduates, or postgraduate students. Less than 10 per cent of the students admitted had only a grade school education. Table V shows that while the number of students does not allow for statistical scrutiny, certain generalizations may be made. The average

TABLE IV

EDUCATION	NUMBER OF STUDENTS ADMITTED
Grade school	19
High school	115
College and postgraduate	135
Total	269

TABLE V

ANALYSIS OF GRADUATES

PREVIOUS LABORATORY EXPERIENCE	GRADE SCHOOL		HIGH SCHOOL		COLLEGE AND POST-GRADUATE	
	NO.	GRADES (%)	NO.	GRADES (%)	NO.	GRADES (%)
None	1	83	39	88	57	92
Up to 6 months	2	88	29	89	47	92
7 to 12 months	2	85	5	89	14	91
Over 12 months	0	0	7	91	2	92

TABLE VI

ANALYSIS OF NON-GRADUATES

PREVIOUS LABORATORY EXPERIENCE	GRADE SCHOOL		HIGH SCHOOL		COLLEGE AND POSTGRADUATE	
	NO.	GRADES (%)	NO.	GRADES %	NO.	GRADES (%)
None	12	54	25	59	10	72
Up to 6 months	2	65	3	71	4	77
7 to 12 months	0	0	6	70	1	86
Over 12 months	0	0	1	99	0	0

Average number of weeks of study: 4.1

TABLE VII

RATINGS OF TECHNICIANS	NUMBER	AVERAGE GRADE (%)
T-1 Master technician	0	0
T-2 Expert technician	1	98
T-3 Senior technician	122	93
T-4 Technician	72	87
T-5 Junior technician	10	82
Students dismissed as T-5	3	70
Students dismissed no rating	48	60
Students transferred T-5	1	92
Students transferred no rating	12	85
Total	269	

grades of the students seem to vary directly with the degree of previous education. In regard to previous laboratory experience, this factor is of considerably less significance than the amount of education in determining the progress of the student in our laboratory course. Table VI is shown merely for the sake of completeness and, since it includes among the nongraduates, students who were dropped due to illness, no conclusions will be drawn. Table VII shows that these latter students did very well up to the time of their transfer. The majority of the students graduated received a Technician 3rd Class or Senior Laboratory Technician qualification on their certificate.

Each of the ratings listed in Table VII denotes certain definite capabilities and qualifications, although it must be remembered that we only recommend the graduates for these ratings. These ratings may be summarized as follows:

MEDICAL LABORATORY TRAINING QUALIFICATIONS*

"*a*. Junior Laboratory Technician, 5th Class: A graduate of the course for laboratory technicians, Army Medical School, or an equivalent course in a civil institution or the equivalent in practical experience, and able to perform the following:

(1) Cleaning and sterilization of laboratory glassware and equipment under direct supervision.

(2) Ordinary routine laboratory procedures, such as urinalysis, blood counts, preparation and staining of slides, and care of cultures.

(3) Care of laboratory animals

(4) Preparation of routine culture media.

b. Laboratory Technician, 4th Class: Qualified as in *a* above, and able to perform the following:

(1) All routine tests on blood, urine, sputum, stools, and pus exudates.

(2) Elementary bacteriology, agglutination and serological tests.

c. Senior Laboratory Technician, 3rd Class: Qualified as in *b* above, and able to perform the following:

(1) Preparation of all stains, solutions, and media.

(2) Routine procedures in any department of a corps area or similar laboratory.

(3) The preservation, preparation, and mounting of pathological materials.

d. Expert Laboratory Technician, 2nd Class: Qualified as in *c* above, and able to perform the following:

(1) Train laboratory technicians in lower grades in the performance of routine laboratory tests.

(2) Assume technical charge of one or more divisions of a corps area laboratory or equivalent.

(3) Carry out, under supervision, more complicated laboratory tests in serology, chemistry, and pathology.

e. Master Laboratory Technician, 1st Class: Qualified as in *d* above, and able to perform the following:

(1) Special ability in one or more laboratory specialties, demonstrated during long and excellent service.

(2) Ability to take charge of any corps area laboratory or equivalent under direct supervision of an officer."

*-MTP 8-1 Medical Department Mobilization Training Program, Medical Field Service School, Carlisle Barracks, Pa., 1940, pages 16-17.

SUMMARY

1. An outline for teaching a laboratory technician course to enlisted men of the United States Army has been presented. This course has been condensed to meet the present emergency. By relying mainly on practical application and demonstration, this entire course is given in the allotted twelve weeks.

2. An analysis of the students graduating during the first twelve months reveals that the final average of grades and individual recommended ratings depend more on their educational background than on previous practical laboratory experience. The majority of students taking this course have been recommended as senior laboratory technicians on the basis of their practical knowledge and ability acquired in this work.

We wish to acknowledge the assistance given by Technical Sergeant A. J. Babalonis in compiling the statistical material.

THE GENERAL MISSION OF MILITARY AVIATION MEDICINE*

BRIGADIER GENERAL DAVID N. W. GRANT, M.C., THE AIR SURGEON, ARMY AIR FORCES

AVIATION is a comparatively new field towards which man has come to direct his energies. At first only a dream, it soon became a reality. Today it is the hobby of thousands, as well as the bread and butter to thousands more. To many it is a curse because it brings fear, destruction and death as an instrument of war. Even under the best conditions it is a calling not without its hazards, for many deaths and broken bones have been left in the wake of its progress, but it has nevertheless advanced rapidly and today can take its place among other occupations as a vocation.

Man has ever been seeking improved methods of transportation, the goal always being increased speed. Each development and improvement of means of travel have presented new medical problems. The development of aviation has been no exception. It has brought with it problems of oxygen want, acceleration, unprecedented demands on the special senses, the nervous system, the heart, and other organs.

World War 1 stimulated the first intensive interest in the medical problems involved in aviation. When the United States entered the War, it benefited greatly by the very unfortunate experiences suffered by the British in the first two and one-half years of that conflict. An analysis of Great Britain's flying casualties during that period showed that of one hundred air fatalities, only two were killed by the enemy, eight deaths resulted from defects in the plane, and ninety were due to deficiencies in the individual, either physical or mental. In those early days virtually no attention was paid to the selection of the individual who was to fly from the medical standpoint. Men were often assigned to aviation duty after they had become unfit for service in the ground arms. The wastage of personnel and material, of course, was enormous.

*Read at the 49th annual meeting of the Association of Military Surgeons of the United States, Louisville, Ky., October 29-November 1, 1941.
Reprinted from The Military Surgeon 90: 281 (March), 1942.

Upon the entry of the United States in the War, the Medical Department was faced with the problem of overcoming all those conditions affecting the physical fitness of the man who was to go in the air. It was at once realized that everyone should not fly, and investigations were immediately begun and have been continued ever since on the problems of selection of flying personnel, and, after their selection, their maintenance in a normal condition.

The general mission of aviation medicine, that of the selection and care of flying personnel is and always has been intimately associated with National Defense. That this mission has been well accomplished is proved by the recent recognition of aviation medicine as a specialty. It is not anticipated that this mission will change, nor is it to be assumed that there will be other than minor difficulties encountered in its accomplishment even when expanded to the present scope of National Defense. *The problem of the moment, therefore, is an orderly expansion of aviation medicine in step with National Defense.* The attending difficulties encountered in any such tremendous expansion are many, but, in a general way, they fall into one of two large groups: First—Problems of personnel. Second—Problems of training.

These problems are tremendous and call for a separate discussion, not within the scope of this paper; rather will I undertake briefly to discuss the general mission of military aviation medicine, that is the selection and care of the military flier, for at this time we are faced with the tremendous problems of securing 30,000 new pilots each year, which calls for the special examination of approximately 120,000 young men. Not only must these men be chosen for their ability to learn to fly, but also from the standpoint of their ability to withstand the stress of operational flying over a long period of time. These figures do not take into account the selection of other crew members, bombardiers, navigators, gunners, and the like, totaling another 100,000.

In selection, the flight surgeon endeavors to select only those individuals for flying training who are most free from real or potential physical and mental abnormalities. The physical examination for flying is more than an objective physical survey. It is an investigation of those bodily and mental attributes, which, by reciprocal action, serve to define the individual as an entity. It has for its object an estimation of his special suitability for a task in a new environment.

Flying subjects man to unfamiliar environmental factors and places under his control a strange and powerful mechanical force which greatly taxes his resources. It creates thereby a new experience which is capable of providing an extraordinary test of his adaptability. The estimate of aptitude for new and specific tasks relies on an evaluation of the individual's entire equipment. It seeks to weigh the potentialities of body and mind and to apply them to the demands of the special situation. It considers native and acquired resources, and computes their utilization. The new task has its origin in the multitudinous needs and desires of man in his peculiar environment. It signifies a new experience, which, found useful and desirable, is retained and perhaps modified for the benefit of the individual and others. It may range in variety from the most simple to the most complex, from the elementary forms of performance to the technical accomplishments developed in the various arts and sciences.

The complexity of the task to the individual is gauged by the degree of stress which it imposes upon his resources. He may have been endowed with sufficient inherent capacity to achieve success, if he so wills, or his capacity may be so limited that achievement is impossible in spite of effort. It is, therefore, obvious that each individual is not qualified by inherent capacity or aptitude to perform the same task with an equal expenditure of endeavor and a like effectiveness.

Aviation is a complex task. It contains the finesse of an art and the exactness of a science. From a military standpoint, it should be performed with the highest degree of efficiency.

The individual for military aviation should be selected with great care. Physical fitness to combat the stresses of flying is not the only requisite. Stability of mental and nervous organization is equally important.

Experience in military aeronautical training has often demonstrated that not every student can meet the requirements of the flying task within the accepted standards. Although he has been physically qualified, is a member of the optimum age group, and has the essential educational background, he may nevertheless fail in adjustment and performance.

It must be remembered that our present physical standards for military flying are based on past experience in matters of efficiency and safety. It is known that some men with physical handicaps, such as having only one leg, or one eye, tuberculosis, or other handicaps, have made excellent pilots. However, immediate efficiency and ultimate economy unquestionably indicate the wisdom of admitting to training only the very best physical material.

In approaching the problem of selection from the psychological standpoint, it must be remembered that the term psychology does not apply to anything mythical, supernatural, or even out of the ordinary. Psychology today deals with very common aspects of human behavior, and the factors that will be mentioned here are the doings, the feelings, and the characteristics of mankind in general, all of which can be observed in ourselves and in our associates with whom we come in daily contact. That individuals differ markedly in general intelligence, in learning ability, in perceptual and memory faculty, and in emotional balance is a fact well known to us all. Those of us who have had to do with the selection of flying personnel have long been seeking to determine just what psychological make-up is necessary for military aviators. Time permits only a brief discussion of these aspects which are especially important in relation to flying.

Intelligence. When we refer to intelligence, we usually mean the capacity to deal effectively with new situations and to understand new problems by applying the results of past experience. Certainly flying is a novel situation for a human being, and in order that he may adequately meet the many problems involved, it is essential that he possess a satisfactorily high level of general ability. But experience has shown that not all individuals having normal or even superior intelligence make successful military aviators. Intelligence is a complex of many functions and two individuals having the same level of general ability may differ tremendously in the amounts of specific abilities which they possess. Learning to pilot an airplane demands a good capacity for learning, but it is learning of a specialized sort and differs considerably

from the ability to learn Greek or mathematics. At least normal intelligence should be required in all flying students. Those with less than this endowment are slow to understand instructions, do not remember, and have great difficulty in solving their problems quickly and effectively. But we need to know more about the individual's mental make-up than merely his general level of ability.

Learning and Memory. To become a military flyer, one must learn a great many things. Normal rapidity of learning and habit formation are essential if progress is to be satisfactory. As in all other things, individuals vary in their capacity to learn. Some learn quickly and forget right away; others learn slowly but remember for a long time. Undoubtedly, the person who learns quickly and retains well the specific knowledges, skills, and attitudes of the types required for military aviation will inherently be the best qualified.

As a result of learning meaning is given to one's experiences. This understanding and interpretation of stimulation of the senses are known as perception, and simply mean that we understand a situation at a glance because we have learned the meaning of this and that stimulus. Good perception is of the utmost importance to the pilot; in fact, it is absolutely essential. He must use his space-perceiving apparatus almost continuously and to the greatest degree. He sees the ground below, and with experience learns much and perceives many things that are meaningless to one who makes his first airplane trip, such as approximate altitude of the airplane, character of the terrain, direction of the wind, and others.

In flying the time factor is all-important. The speed with which the various maneuvers must be learned and co-ordinated is definitely related to the speed of the aircraft. Many of the responses of the pilot must be learned so well that they become almost automatic in nature, and not just a memorized method of movements of stick, rudder and throttle. Extreme delicacy and discrimination are essential, correct interpretation is demanded, and sound reaction must be initiated with a maximum of speed. When material is poorly learned, or when one's memory errs or falters, one cannot effectively respond, and too often penalties are exacted for failure. It should be apparent then, that normal rapidity of learning and habit formation is necessary in a flyer. This includes a plastic learning faculty to permit quick formation of association pathways, giving correct and successful reactions.

Attention. As we continue considering psychological processes, we note that many of them are closely related, that they often are distinguished from other processes with difficulty, and that they influence each other in various ways. Attention, for instance, has much to do with effective learning, for in order to take instructions well it is necessary to be able to keep concentrated on the subject at hand. Modern airplanes are built for high performance, built with more than one engine, variable pitch or constant speed propellers, retractable landing gears, de-icers, radio and numerous instruments pertaining to engine and flight performance. All these apparatus must be attended to by the pilot, and probably in no occupation is it more important to have just the precise development of attention for that particular job than in piloting an airplane.

The desirable type of individual must have this faculty of attention developed to the proper degree—a too highly developed degree of concentration may even make a dangerous flyer. Such an individual becomes so absorbed in concentration on one particular thing until that goal is reached that he is oblivious to everything else. Individuals prone to "absent-mindedness" are usually concentrating so entirely on something else that they can pay no attention to the problem at hand.

On the other extreme is the distractable individual, who seems to lack the capacity to concentrate at all. His attention is so susceptible that it will not remain fixed long enough for him to comprehend the meaning of any of the stimuli he receives so rapidly. Normal span and control of attention is certainly one of the requirements for a flyer.

Emotion. Perhaps there is no vocation in which the emotional factor plays a more important part than in aviation, for when man is flying he is completely out of his element, surrounded by countless dangerous situations, his life and that of his passengers depending upon an emotional stability which will enable him to react at times with lightning-like rapidity. It is well known that resentment, irritation, anxiety, surprise and many other emotional states retard the thought processes and interfere with normal voluntary control over our co-ordinated movements. While under the influence of an emotion, one may make mistakes and errors of judgment which would not have been made under other circumstances. It is even conceivable that one may become "paralyzed" with fear.

The emotionally unstable pilot is apt to carry financial, family or other troubles into the air with him, with the result that he is preoccupied and inattentive. The question of emotional stability is extremely important and flying personnel should have above the average emotional control.

Reaction Time and Age. The regularity and stability of an individual's reaction time are as important in flying as in any other calling where quick decisions and actions are required. The time factor is so all-important in the flying game that those individuals who are even a little slow in their thinking and actions are at a distinct disadvantage. Reaction time may be modified by many causes, such as infectious diseases, fatigue, and other factors. However, aside from such conditions, individuals vary greatly in their speeds of reacting. The question of reaction time has held a prominent place in studies related to the problem of flying for many years.

Youth is a desirable characteristic of the flyer. Reaction time becomes slower as we grow older, though this slowing down process varies in different persons. With increasing age, particularly in postprime years of life, there is a lessened plasticity of the learning faculty. Habits of long duration guide and fix much of one's activity, and new ways and things are assimilated with difficulty. Age should be given a very definite consideration in selecting those who are to fly.

These then are a few of the psychological phases of aviation which we consider in the selection of the individual who is to fly. We should select only those individuals who have a fortunate combination in desirable degrees of the psychological factors we have here discussed, and such individuals will

not be found to be rare. No doubt many hundreds of thousands of ordinary adults are well equipped as potential military flyers, but it should be remembered that there are others, in not insignificant numbers, who for psychological reasons should definitely not fly.

Maintenance. Maintenance of personnel is not a matter which is peculiar to the Air Force; however, in addition to the usual procedures such as hygiene, sanitation, diet, rest and relaxation, which are essential to all, there are certain specific problems which are unique to aviation.

Therefore, in any logical discussion of the subject it will be necessary to mention only briefly some of the problems that we, associated with aviation medicine, are vitally concerned with as having a direct bearing on the maintenance problem:

1. OXYGEN AND HIGH ALTITUDE FLYING

The composition of the atmosphere is uniform below the stratosphere, i.e., approximately 70,000 feet.

	BY VOLUME
Oxygen	21%
Nitrogen	78%
Rare inert gases	1%

As air is compressible, it is denser at lower than at higher levels so that the decrease in pressure on ascent in 1,000 feet is greater near the ground than it is, for example, at 20,000 feet. At 18,000 feet pressure has fallen to half that of the ground level.

Partial pressure in a mixture of a number of gases is that pressure exerted by any one gas within the mixture. It is proportional to the percentage of that gas in the mixture. Oxygen being approximately one-fifth of the atmosphere by volume exerts a partial pressure of 760/5 mm., which equals 152 mm. at ground level.

Oxygen saturation of the hemoglobin of the blood depends on the partial pressure of the oxygen in the lungs. At ground level the partial pressure of oxygen is sufficient to nearly saturate the blood leaving the lungs, so that taking extra oxygen at ground level for short periods, while doing no harm, does no good except in certain instances. Between 15,000 and 18,000 feet the atmospheric pressure has fallen to 428 mm., and the oxygen partial pressure in the lung to approximately 50 mm. of mercury. When the partial pressure of oxygen reaches 50 mm., the hemoglobin can only be approximately 75 per cent saturated, and the tissues are unable to get sufficient oxygen.

Lack of oxygen affects all tissues of the body, and exposure to even slight degrees of anoxia too slight to produce immediate effects will in time impair efficiency and lead to unnecessary discomfort and fatigue.

a. *The Brain. Psychological.*—Defective judgment, spurious self-confidence; lack of self-criticism; inaccuracy; lack of appreciation of time, and the like.

Reasoning.—Alertness dimmed, all mental processes slowed down, later unconsciousness comes on without the individual being aware of anything abnormal.

b. *Vision. By day* the light may appear dimmed and acuity is dimmed.

By night adequate oxygen is of even greater importance because even the slight degree of oxygen lack at 4,500 feet considerably impairs the ability to adapt one's eyes to dim lights and to discern objects dimly illuminated.

c. *Cold.* Lack of oxygen causes the hands and feet to feel cold, and if this occurs, supplying oxygen brings a flood of warmth within a few minutes. However, extra oxygen will not prevent one's feeling cold if one is inadequately dressed.

It should be noted that the above changes, at least in the earlier stages, are not appreciated by the individual himself. Even syncope is such an imperceptible process, that the subject on recovering consciousness may be completely unaware he ever lost it.

Our Air Force now requires oxygen to be used as follows:

a. All flights of 10,000 feet over one hour's duration.
b. All flights to 15,000 feet regardless of duration.
c. From the ground up when the rate of climb is 2,000 feet per minute.
d. Night from ground up.

2. BENDS OR DECOMPRESSION SICKNESS

When air is breathed into the lungs, oxygen alone is absorbed by the blood cells, but by the simple physical laws all gases pass into solution in the fluid plasma of the blood proportionate to their partial pressure. Thus the nitrogen in the plasma is in equilibrium with nitrogen in the air at ground level. During ascent, the pressure of nitrogen in the air, of course, falls and nitrogen comes out of the blood in just the same way that carbon dioxide comes out of a soda syphon when the pressure is reduced by opening the tap. If ascent is slow, the nitrogen has time to diffuse through the lungs. If, however, the ascent is rapid to great heights, the nitrogen comes out of the blood and forms bubbles in the vessels and tissue fluids, causing the tearing of the tissues and blockage of the vessels. This discovery was first made when investigating the caisson disease, or bends, in divers. Divers at 200 feet are exposed to a pressure of six atmospheres, and, while at this depth more nitrogen is absorbed in their body fluids, as rapid ascent takes place they suffer from bends, but slow decompression will completely prevent the illness. A similar condition may develop when ascent is made to 30,000 feet or more at rates of climb attained with the modern aircraft.

3. ACCELERATION OR ACTION OF CENTRIFUGAL FORCE ON PILOTS

Any aircraft flying along any arc of a circle, whether produced by pulling out of a dive, a tight turn, a diving spiral, or any combination of fighter aerobatics, has acting upon it from the center of the circle a centrifugal acceleration which varies directly as the square of the linear velocity and inversely as the radius of the circle. Weight is a force and is expressed as the product of the mass and the acceleration due to gravity or "G." Hence, when a pilot has acting upon him an acceleration of several times that of gravity, his weight increases in the same proportion: and at a centrifugal acceleration of seven times "G" a pilot weighing 180 pounds normally, weighs 1,260 pounds. Every tissue in the body takes part in this increase in weight. At approximately

seven "G" the blood becomes as heavy as melted iron. Consequently, the weight of the hydrostatic columns of blood, which has to remain unaltered in order to maintain the circulation through the brain on the arterial side and the inflow into the heart on the venous side, increases to an extent that cannot adequately be met with by the venous mechanism which returns the blood to the heart from the abdomen and legs and in the capillaries of the abdominal organs and leg muscles where the cross-sectional area is very large. As a consequence of these circulatory changes, there is a fall in the blood pressure in the large vessels supplying the brain and eyes, and, when this reaches a given value for the individual pilot, failure of vision occurs, which we call the "black-out," and if this force continues for an appreciable length of time, unconsciousness will ensue. It has been constantly noted that a succession of "black-outs" without impairment of consciousness is followed by undue feeling of lethargy, lassitude, impairment of mental concentration, or, in other words, extreme fatigue. Average young pilots in good health will take 4½ to 5 "G" for four seconds without a black-out.

4. OCCUPATIONAL FATIGUE

The word "fatigue" here is used to describe: (a) A complex and rather vague clinical condition which is often thought to constitute a definite disease entity, whenever a subjective sense of tiredness predominates. (b) Intracellular or other tissue changes, resulting from overstimulation.

Occupational fatigue has been a subject of paramount interest to physicians interested in industrial medicine and to medical officers in the military services for a great many years. Its presence greatly diminishes both the quantity and quality of the work rendered by the worker in industry, or the military man in one of the armed forces. At no place in the military service is fatigue so likely to occur, and if it does occur, it is not of such importance as in the Air Force.

The term "fatigue," when used in connection with flying, has come to be attached to a syndrome found in flying personnel, particularly those engaged in operational or combat units. Its manifestations are the effects of such work upon the normal individual being submitted to an abnormal amount of stress. Many terms have been used to describe the condition, such as aeroneurosis, flying stress, operational fatigue, staleness and others. I prefer to use the term "flying fatigue" as it seems to me that this is the most descriptive—any change from the normal can thus be associated with the etiology of the conditions.

Nervous breakdown in pilots has been noted since the early days of flying. In military aviation, where the pilot not only must handle airplanes powered with one or more motors of extremely high horse power and be subjected not only to the effects of the resulting vibration, speed, rapid changes in altitude, but also while managing this machine, to the stress of close formation flying and fighting in the air, fatigue makes its appearance much earlier than when flying for pleasure in commercial airplanes. It must also be kept in mind that the lowered efficiency resulting from fatigue in military flying personnel is very likely not only to affect the individual himself, but also to endanger

indirectly the lives of all those who may be associated with him. Many of our military combat planes of today carry crews of from three to ten men. In carrying out a military operation, exact timing and co-operation on the part of every member of the combat crew are necessary, and the results obtained are not likely to be any better than the efficiency reflected by the weakest member of that crew.

The "Flight Surgeon" of the Army is charged with the *selection* and *maintenance* of flying personnel from the physical standpoint. In the selection of applicants for flying training, he attempts to select only those who not only meet the high physical standards necessary for arduous military field service but also only those who possess the necessary psychological qualifications to enable them to complete flying training and to withstand the flying stress or "occupational stress," which will be imposed upon them to a severe degree by combat flying. If proper selection is made, maintenance of the individual will likely not become a difficult problem; however, our biggest problem in maintenance is flying fatigue. The variability of the relative proportions of the subjective and objective symptoms of flying fatigue renders it difficult to diagnose them in the early stages. The trained flight surgeon will be on the lookout for the early and insidious symptoms of this weakness so as to institute prophylactic measures against its full development, with resulting total loss of the individual to combat flying.

PHYSIOLOGY OF FLYING*

Hazards and Remedies

Lieut. Col. D. Bruce Dill, Dayton, Ohio

FLYING is a hazardous undertaking, and it is fitting to discuss here those hazards that are physiological in nature. It has been popular to think of *anoxia* as the chief hazard of high altitude flying. While it is no longer of paramount importance, it still deserves some attention. The man breathing air at sea level is able to saturate his blood almost completely with oxygen. The range of saturation is remarkably narrow, usually being within the limits of 94 to 96 per cent. This results in a partial pressure of oxygen in arterial blood of about 80 mm. Hg. Man has become accustomed to that condition; the tissues of the body have been organized on the basis of that partial pressure of oxygen in blood coming to them. The one organ most sensitive and which suffers greatest from reduction is the brain. This hazard of oxygen lack arises between 10,000 and 20,000 feet where the pilot is not using his oxygen supply. As he goes higher, using his oxygen supply, even assuming it is functioning perfectly, when he reaches an altitude of about 37,000 feet, he again begins to become

*Lecture XXI, reprinted from *Collected Lectures of the Metropolitan State Hospital*, Waltham, Mass., 1942.

anoxic. The reason is commonly overlooked by medical students and physiologists who do not appreciate the fact as the total pressure decreases a point is reached at which even with pure oxygen the blood is not saturated. This critical altitude is around 37,000 feet, and breathing oxygen at 40,000 feet is roughly equivalent to breathing air at 10,000 feet. The limit of consciousness breathing pure oxygen is near 45,000 feet.

Another hazard and one much less well understood is *aeroembolism*. It depends not upon oxygen supply but upon the absolute pressure. At 18,000 feet the pressure on the body is just half an atmosphere, and at 40,000 feet it is one-fifth of an atmosphere. As a result, nitrogen tends to be released from solution in body fluids. As we sit quietly at sea level we have in our lungs a partial pressure of nitrogen of something over 550 mm. That gas in the lungs must necessarily be in equilibrium with the blood passing through the lungs, and the solubility of nitrogen in the blood is such that about 1.4 volumes per cent of gaseous nitrogen is dissolved in it. Every liter of the blood will contain about 14 c.c., and that is also true of the water in the rest of the body. A larger proportion dissolves in body fat. The analogy has been drawn between the state of this gaseous nitrogen in the body fluids and the state of carbon dioxide in a bottle of soda water. In either case, when the pressure is suddenly decreased, gas bubbles form.

There are a number of curious facts about aeroembolism that we do not understand. We know that at 18,000 feet the pressure on the body is reduced one-half, and yet no one ever experiences any trouble from aeroembolism here, nor even at 25,000 feet. At 30,000 feet very few people will experience symptoms during the first hour of exposure. At 35,000 feet no one is likely to suffer during the first few minutes.

The best guess as to the explanation of these facts is that while the nitrogen escapes from solution at once, the first bubbles formed are too small to cause trouble. In time they increase in size by coalescing with adjoining bubbles by the diffusion into them of carbon dioxide and water vapor oxygen.

A third hazard is that of *acceleration*. We know that a man may faint on a hot day while standing at attention, i.e., when exposed to 1 "G." When we consider that a man in an aircraft is not uncommonly exposed to three times that force and for short times to five or six times that acceleration of gravity, we can readily imagine that fainting is very likely to occur. In a pull-out from a dive, the "G" is often as great as 5 for a period of a few seconds. If the man is sitting erect, venous return from below the heart will be stopped, and the arterial supply above the heart, and in particular to the brain, will be handicapped. Within a short time the heart is inadequately filled. There will be but a small amount of blood discharged with each beat, and the tendency will be for blood that does leave the heart to go down rather than up. Most of our knowledge of the physiology of acceleration has come from Germany, where a centrifuge has been in use for some years. There are many important questions awaiting solution, such as the possibility of selecting men who are resistant to acceleration, of determining the factors that make him either more or less resistant to acceleration, and of training men in the techniques of resisting acceleration.

A fourth hazard of flying is *cold*. This needs no special emphasis here, because the hazard of cold in high altitude flying is little different from that experienced by the man on the ground in cold climates. One point requires special mention—the relation of shivering to the problem of oxygen supply. In the present oxygen-supply systems it is assumed that the man is not going to use very much oxygen. However, if the man gets very cold and starts shivering, his oxygen consumption then may go up to two to five times, and then two hazards may be superimposed, anoxia developing if his oxygen system is inadequate for this emergency.

Vibration can be put down as a fifth hazard. This properly includes noise. Some individuals are extremely resistant to noises. Others are rather sensitive, and it is safe to assume that the exhaustion experienced in flights of many hours' duration depends in no small degree on this hazard. In a project at Harvard designed to study physiological effects of noise, physicists have been able to reproduce the characteristics of the noise of an airplane propeller with extraordinary fidelity.

A sixth hazard which cannot be separated from these others is *fear or anxiety*. It arises from being in a strange environment, from being uncertain whether he is going to reach his field or whether, when he gets back, he will be able to land. If we add to the hazards already mentioned the emotional experience of combat, it is quite clear that the combined effect is a serious strain on the organism, a strain that can be tolerated once or twice or a few times but cannot be tolerated too often; there must be opportunities for recuperation.

Having presented some of the facts about the hazards of high altitude flight, I am going to return now to a more detailed discussion of some of them, beginning with anoxia. There is, first of all, an adaptive response to anoxia that is believed to depend on stimuli arising in the carotid body. Respiratory volume is increased, thus preventing such a reduction in arterial oxygen as would otherwise occur. The extent to which respiratory volume can increase in extreme anoxia has been demonstrated by Dr. Corwin and Mr. Horvath here at the Metropolitan State Hospital last summer. The respiratory volume reached 25 to 30 liters per minute merely from the stimulus of oxygen lack. The same patients, if poisoned with carbon monoxide, would become unconscious without any increase of respiratory volume.

What are the effects of oxygen lack on the muscular system? We know that a single contraction of a muscle can be carried on equally well whether oxygen is present or not. It can be shown that a man at an altitude of 20,000 feet has as great strength as under ordinary conditions. However, if he attempts to continue, there will be a breakdown, because recovery processes are handicapped by oxygen lack.

In the nervous system the story is different. The functioning of the nervous system depends upon a virtually continuous supply of oxygen and almost immediately, when a given stage of anoxia is established, the various mental functions show decreased efficiency. Sleepiness, for example, is one of the common effects of anoxia. A man exposed to moderate anoxia may sleep for hours at a time. With prolonged and more severe exposure, the sleepiness may verge on unconsciousness. Stupor is not uncommon. The phenomenon of persevera-

tion is commonly seen. We have sometimes asked anoxia subjects to write a sentence that includes the phrase "around the rugged rock." It is a common error, when they get to this expression "around the rugged rock," a phrase which in itself suggests repetition, to write repeatedly "around the rugged rock."

If anoxia is prolonged, more unpleasant effects, such as nausea, headache, and vomiting, may occur. The headache may become most severe after the period of exposure, as is also true in carbon monoxide poisoning.

An instructive illustration of both the direct and indirect effects of failure of oxygen supply may be given. The test subject was riding a bicycle in the chamber at 40,000 feet. We had him hooked up to a cardiotachometer, and I was getting ready to make an arterial puncture on the man while riding the bicycle in order to determine the adequacy of his oxygen equipment. I picked up the needle and syringe and started to put a sponge in a dish of alcohol. This dish instead of having the usual dye-colored fluid had a clear solution. I wasn't sure it was alcohol and I took off my oxygen mask momentarily to sniff it. A few seconds later I again took off my mask to announce over the teletalk that I was going to make the puncture. Then I walked around, facing the subject, but as I got into position with the syringe in one hand and the sponge in the other, I slowly settled to the floor and leaned my head back against the wall of the chamber. This, for the first few seconds, did not seriously alarm the man on the bicycle. He thought it was a peculiar thing to do, but in about ten seconds he fully appreciated that I had passed out, and also that there wasn't anything he could do about it. He was hooked up to an oxygen system that didn't permit him to get within reach of me. There was an observer on the other side, who signaled to the chamber operator outside, and within two minutes we were down to ground level. The most interesting observation made in that case was on the man riding the bicycle. His pulse rate was 110 before my collapse; it rose to 120 when he first saw me acting curiously, and then it went to 185 during the next ten seconds, when he appreciated that I had passed out. The lesson from this is that in a plane where you are at a higher emotional pitch than is likely to be reached in a chamber, an accident to one man is certain to have a very powerful effect on some of the other men. That is the time when serious mistakes are likely to be made, leading possibly to disaster.

Such an incident illustrates the sort of experiences that one faces in the air, and I want to emphasize once more that in studying the problems of high-altitude flight, you should divorce from your mind the deep-seated idea that they can all be answered by putting mice in a cage and subjecting them to low oxygen.

Returning now to the subject of aeroembolism, I wish to say a little about protection against it. Since it is produced by the formation and growth of nitrogen bubbles, it would appear reasonable to try eliminating some of this nitrogen before starting a flight. The most effective means consists in breathing pure oxygen during exercise, the nitrogen, of course, being gradually evolved by the lungs. Exercise that raises the metabolism to four times the

resting level, involving a respiratory volume of twenty liters a minute and an increased circulation rate, will result in the release of at least half the nitrogen of the body within a half hour. The man is then able to undergo a greater reduction in pressure or to stand a very much longer exposure to a given altitude than would ordinarily be the case. This procedure is likely to have usefulness in some missions. It obviously cannot be used in defense warfare, because a man on defense has to be ready on a minute's notice. It seems that it could be made practical in offensive operations or whenever missions are planned ahead of time.

The obvious method of protecting against the anoxia and cold, as well as the aeroembolism of extreme heights, is the pressure cabin. You have heard of the commercial plane that maintains a small positive pressure in the cabin so that when it is 20,000 feet, the effective pressure inside is equivalent to 10,000 or 12,000 feet. There is considerable advantage in this. The military pressure cabinet is still on the blueprint stage so far as I know. On paper at least, it is possible to make a cabin that at 40,000 feet will maintain a pressure within equivalent to 10,000 or 12,000 feet. Compression of that air will heat it enough to keep it comfortable.

Something should be said about *visual function* of pilots. A subject about which I presume you have heard much within recent years is night blindness. There was a great furor about two years ago, and one of the first published papers in this field indicated that vitamin A deficiency was rather common. Many measurements have been made during the last year in military personnel, and practically speaking, you never find a case of poor night vision improved by vitamin administration. However, these studies have been useful in revealing a wide variation among healthy men, a variation that appears to be inherited; at any rate, it is relatively constant in a given man. It remains to determine the practicability of selecting men with exceptional night vision for night flying.

SOME PROBLEMS IN AVIATION MEDICINE*

Ashton Graybiel, M.D., Boston, Mass.

Aviation medicine is a newcomer among the medical specialties. It was born during the First World War, when the lack of fitness among airplane pilots became strikingly apparent. Some idea of the urgency of the problem at that time can be gained from the fact that in the British forces 90 per cent of the plane casualties during the first part of the war were due to pilot error, and only about 2 per cent of the plane casualties were due to enemy action.

Steps were immediately taken to improve upon the method of selection of pilots and to do a better job in the maintenance of their health and fitness. Certain criteria were laid down which served as a yardstick for selection. For the most part, they dealt with the physical criteria for selection, and the value of the standards laid down at that time is attested by the fact that they have not been materially altered since.

Following the First World War, aviation medicine received a setback, and interest was not revived until the great development of commercial aviation, which began in the late twenties. At that time various studies were made, not only with regard to the care and selection of pilots, but also with regard to the comfort and safety of passengers. Now the major interest has again shifted back to military aviation, with the onset of the Second World War. At the present time, there are many and important problems requiring solution, and this condition has come about so abruptly that there is a great lack of trained personnel in this field.

Many people at this time are under the impression that the modern airplane has reached such a state of perfection that the pilot's work has, therefore, become much easier and simplified. Actually almost the reverse of this is true. The airplane, as Dr. Hunsaker of the Massachusetts Institute of Technology points out, is still subject to the laws of gravity, will still slip and fall into a spin, and still presents many of the "old difficulties" in flight. With every increase in the reliability of the airplane, there has also been an increase in expectancy of performance. Increasing the speed of the plane and the height to which it may ascend has served to introduce such problems as aeroembolism, anoxia, and the black-out. None of these problems were of any great significance during the First World War, but are of first importance at this time.

THE SELECTION OF PILOTS

Someone has said that out of about thirty-odd billion people born in the world, only about five thousand ever amounted to much, and that is somewhat the way it is with the selection of pilots. Out of a large number of apparently

*Lecture XXII, reprinted from *Collected Lectures* of the Metropolitan State Hospital, Waltham, Mass., 1942.

healthy individuals, only a small number are really fitted, both physically and psychologically, to pilot an airplane. Armstrong has stated that if a man is examined and is found to have no obvious physical defects and is satisfactory for any ordinary type of occupation, he still could have a hundred defects which might disqualify him from becoming a good pilot. In addition to the physical defects there are, of course, the psychological aspects to be considered, and the pilot's aptitude for flying.

Some idea of how few are fully qualified for military pilots is gained from a study of the pilot selection at one of the training stations. The majority are selected from the students in nearby universities, although a number apply from all over the United States. First of all, these college students are sent a questionnaire, which they fill out; if certain basic educational and physical requirements are met with, they are given the physical examination. This examination is quite thorough. It not only includes the usual physical examination but also includes a careful examination of the special senses and a "short-arm" psychological examination. As a result of this procedure in a typical sample of 753 candidates, only 42 per cent were accepted as representing sufficiently good material to start training. Thus, in an already select group of individuals, about 60 per cent were immediately disqualified. Of those that are finally selected as student pilots, somewhere between 25 per cent and 40 per cent are eliminated during the first six months of training. This represents, of course, not only a large economic loss, but, what is more important in wartime, a serious loss of time and effort.

A chief problem then is to devise some system of selection whereby only those who are likely to succeed will be permitted to enter training. The problem has been attacked in various ways. For example, the physiological and psychological characteristics of successful pilots have been analyzed in an effort to obtain criteria for selection. Another method has been to carry out various examination procedures and tests on all the incoming pilots and then see if those failing and those succeeding make different scores on the various tests. It is not to be hoped that any single test will separate the good from the bad material, but it is hoped that some day a battery of tests will be discovered which will greatly aid at least in the selection process.

Much time and care are now given to the maintenance of health and physical fitness in pilots. Periodic examinations now are required in order to insure that certain physical requirements are being met. In commercial aviation a limit of eighty-five hours a month has been set as the maximum flying allowed. Gradually, there has come about an increasingly close relationship between the pilot and the flight surgeon, with the common objective of keeping the pilot flying as long as possible.

OXYGEN LACK

One of the most important problems in aviation medicine results from the progressive decrease in oxygen pressure with increasing elevation. Every physiological function depends upon the supply of oxygen. and when this is reduced, the function must be restricted accordingly. The extent to which the body is affected depends not only on the altitude reached but also on

the rate of ascent and the length of time aloft. Certain physiological characteristics of the individual are important as is the state of health. As Dr. Dill has pointed out, the aviator or airplane passenger makes certain adjustments as the result of reaching a high altitude, but, unlike the mountaineer, he undergoes little or no acclimatization even though oft-repeated flights are made.

Commercial airplanes for the most part fly at altitudes below 10,000 feet but in certain regions they fly at altitudes of 14,000 to 15,000 feet and may on occasion go even higher. A recent Civil Air Regulation requires the crew to use additional oxygen if flying above 12,000 feet or if flying above 10,000 feet for thirty minutes or longer. Nearly all the commercial planes which fly at altitudes over 10,000 feet contain equipment for oxygen administration to passengers. In military aviation most of the flying is still done at relatively low altitudes, but some is done at elevations between 35,000 and 40,000 feet. At 40,000 feet the alveolar oxygen tension of a pilot inhaling pure oxygen corresponds to that of a pilot breathing air at 11,000 feet. From a practical standpoint it is nearly impossible to prevent slight leakage when using an oxygen mask, so that the degree of anoxemia usually exceeds the theoretically calculated amount.

Very briefly I will discuss some of the effects of oxygen lack, particularly on the nervous and circulatory systems.

If anoxia is rapidly produced, as in an actual or simulated ascent in an airplane, the first and most important effects are seen in relation to the central nervous system. Psychological impairment to a slight degree may be observed below 10,000 feet but it becomes more definite at higher elevations. At first there may be a feeling of well-being and even euphoria. A false sense of enhanced powers is often experienced, which is in many respects similar to mild alcoholic intoxication. This state gradually passes into one of mental dullness at elevations around 14,000 to 15,000 feet. and some persons become sleepy. At this stage of decreased sensory acuity vague feelings of uneasiness and bodily discomforts tend to disappear, the anoxia acting somewhat as an analgesic. At higher elevations the signs and symptoms of anoxia become progressively more marked, and fainting or unconsciousness occurs. Few, if any, unacclimatized persons remain conscious at elevations in the neighborhood of 25,000 feet.

The obvious treatment for symptoms of anoxia, or better yet, their prevention, rests in supplying an additional amount of oxygen. The most practical method of giving additional oxygen is by means of a mask fitted over the nose and mouth. This procedure is quite satisfactory for elevations up to 35,000 feet or a little higher, but above that level the partial pressures of water vapor and carbon dioxide in the alveoli form too large a part of the total pressure and even breathing 100 per cent oxygen is insufficient. It then becomes necessary to supply oxygen under pressure as in a supercharged cabin plane.

My own interest in aviation medicine is concerned chiefly with problems relating to the cardiovascular system. In considering this problem, it is essential to distinguish between the direct effects of oxygen lack on the heart and the effects on the peripheral circulation both in the healthy person and in the patient with heart disease.

With regard to the heart itself there is much evidence that a person with a normal or even a diseased heart may withstand surprisingly severe degrees of oxygen lack without evidencing heart failure.

Healthy subjects during high altitude flight or those exposed to low oxygen tensions in the laboratory rarely complain of symptoms directly referable to the heart.

The dyspnea which may be noticed is not severe and is due to stimulation of the peripheral chemoreceptors by anoxemia and is not a sign of heart failure. Pain of cardiac origin is never noticed and palpitation very rarely. The fatigue which commonly appears during long flights, and the state of easy fatigability which follows, is probably related to the effect of anoxia on the central nervous system.

Of all the circulatory changes due to diminished oxygen tension, acceleration of the heart rate has been studied the most. With successive decreases in oxygen tension the rate continues to increase slowly, but the increase in rate is not marked until extreme degrees of anoxemia are reached. Sometimes there is little change in the heart rate or actual slowing and bradycardia may be associated with the collapse.

The changes in blood pressure due to increasing anoxia follow several fairly well-recognized patterns. In the majority of healthy persons there is little change until the oxygen in the inspired air has fallen to low levels. Then there may occur a gradual rise in systolic and a fall in diastolic pressure, with consequent increase in pulse pressure. In susceptible persons exposed to moderate degrees of oxygen deficiency, fainting may occur and there is a sudden fall in both systolic and diastolic pressure. This fainting reaction is in the nature of peripheral vascular collapse and is caused by cerebral anoxemia and is not due to heart failure. In a few subjects there is little change in blood pressure until unconsciousness supervenes. It is only at this stage of crisis that the fall in blood pressure may be directly attributable to a failing heart.

The cardiac output increases in healthy persons at rest under conditions of moderate anoxia. This increase may be regarded as a compensatory or adjustment mechanism. With marked decreases in oxygen tension the cardiac output may fall as a direct result of cardiac anoxia, but this stage is never reached under present flight conditions. More studies along this line are necessary before the relationship between anoxia and cardiac output is properly understood.

There are many reports, especially in the older medical literature, describing cardiac dilatation or hypertrophy in mountaineers or in those resident at high altitudes. There are also a few published observations purporting to show that cardiac enlargement may occur in aviators or in subjects exposed to reduced barometric pressures. Etienne and Lamy observed slight to moderate cardiac hypertrophy in ten of the eleven aviators of a celebrated escadrille. They considered that this hypertrophy developed in two stages and invariably involved the left ventricle. In the first stage, which lasts only a few months, the heart increases in size relatively rapidly, and this is followed by a second stage lasting two to three years, in which the hypertrophy is slowly progressive.

Whitney observed dilatation (by percussion) of the heart in five of ten healthy subjects as a result of a reduction of barometric pressure simulating an altitude of between 14,000 and 20,000 feet. Increases in transverse diameter of from 3 to 5 cm. were said to occur. Le Wald and Turrell, on the other hand, in a carefully controlled roentgenological study of 89 aviators under conditions simulating high altitude, observed a decrease in size of the heart in 14, no enlargement in 66, and an apparent enlargement in 9. In 6 of these 9 the increase in size was explained by alterations in the position of the diaphragm while in the remaining 3 the transverse enlargement was only 0.4 cm. or less. It is now quite generally agreed that the normal heart, if the subject is at rest, does not dilate even with greatly lowered oxygen tensions. This conclusion is borne out by animal studies in which it is shown that cardiac dilatation does not begin until the oxygen saturation has fallen to about 50 per cent or below.

The electrocardiographic changes have been studied during actual flight conditions as well as under conditions simulating high altitudes in the laboratory. In healthy persons these changes consist of progressive lowering of the S-T segments and T waves with increasing anoxia. Occasionally, the T waves may be diphasic or even slightly inverted. Some of the most marked electrocardiographic changes observed in healthy subjects were obtained by Dr. Corwin and Mr. Horvath in experiments on acute anoxia conducted in this hospital. Especially marked was the lowering of the S-T segments and T waves.

That alteration in the carbon dioxide tension alone may cause electrocardiographic changes has been described by Barker and others, and we have confirmed these observations as to the lowering of the T waves and slight downward displacement of the S-T segments following overventilation for three minutes. That overventilation may be a factor in producing the electrocardiographic changes observed at high altitudes is highly probable.

Thus, in normal persons the symptoms which may appear under conditions of civil or military aviation and the changes in circulatory dynamics do not indicate heart failure but may be considered in the nature of adjustments or compensatory reactions to the oxygen deficiency; the most trustworthy evidence we have supports the view that the normal heart is not damaged. With respect to peripheral circulatory collapse this may occur in susceptible individuals even at relatively low altitudes and is due primarily to the effect of lack of oxygen on the central nervous system.

DECOMPRESSION ILLNESS

Decompression illness is another hazard which must be considered. When the atmospheric pressure is suddenly decreased, such as occurs during rapid ascents to high altitudes, symptoms arise as a result of the formation of gas bubbles in the body tissue and fluids. This bubble formation takes place in accordance with the laws governing the solubility of gases. Blood passing through the lungs is exposed to the gases in the atmosphere, and each gas is absorbed according to its partial pressure. Relatively large amounts of nitrogen go into solution because of its high partial pressure, while oxygen and carbon dioxide are taken off in smaller amounts. The body has special transport systems for oxygen and carbon dioxide but not for nitrogen, which is relatively inert and obeys the laws of simple solution. Now, if the atmospheric pressure

is decreased, the body tissues and fluids tend to give up the dissolved gases, principally nitrogen, by way of the lungs. If the decrease is sudden and of the order of half an atmosphere or more so that the concentration of nitrogen is at least double what it should be, this gas will come out of solution and form bubbles to which is added some oxygen, carbon dioxide, and water vapor from the surrounding blood and tissues. There is considerable difference in opinion regarding the height of ascent before clinical symptoms appear. Armstrong states that gas bubbles are found in the spinal fluid at 18,000 feet while bubbles in the blood and tissues generally have not been found below 30,000 feet. He believes symptoms are rarely noted below 30,000 to 35.000 feet. However. English observers are of the opinion that symptoms may appear at lower altitudes than these. The most dangerous symptoms occur, of course, when bubbles appear in the blood stream. Aeroembolism of a cerebral artery may lead to severe central nervous system disturbance while if the coronary arteries are involved, heart failure may result. This, then, becomes an important problem, especially in military aviation. Dr. Dill, in the previous lecture, has told you of his striking experiences with aeroembolism.

ACCELERATION

Another adverse influence on the cardiovascular system is that of centrifugal forces (due to positive accelerations amounting to as much as nine times the force of gravity) which may be encountered during the combat maneuvers of modern fighter aircraft. The effects of positive accelerations when these are in the longitudinal axis of the body are observed most dramatically in the displacement of blood from the upper to the lower portions of the body. Forces greater than 4 to 5 "G" acting for three seconds cannot be sustained without symptoms. If the force acts only for a short time, the blood supply to the eyes and then the brain becomes deficient, and the vision becomes dimmed or lost. If the centrifugal forces act sufficiently long, the venous return to the heart becomes inadequate and complete circulatory collapse ensues. Dr. Dill, in the previous lecture, has described the underlying physiological changes and the symptoms produced.

AIRPLANE SICKNESS

Sickness in an airplane is usually due to the movements of the plane or oxygen lack, or a combination of both. Sometimes the former is referred to as airsickness and the latter as altitude sickness, but as both conditions may be present at the same time, a term such as plane-sickness is useful.

Airsickness is akin to seasickness both in etiology and symptomatology. The up and down movements of the plane are the chief immediate cause, but many other factors such as fear, fatigue, alcoholism, etc., play an important role. Persons who develop airsickness readily do not make successful pilots, but on occasion even a pilot of long experience becomes sick.

Nausea and vomiting may be caused by oxygen lack and may be a distressing part of acute altitude sickness. Pilots will often fly higher than usual in order to find less turbulent air. Often a compromise must be worked out between "rough" air and adequate oxygen and "smooth" air and deficient

oxygen. The story is told of a South American pilot who was informed by the steward that only eight box lunches had been put aboard for fourteen passengers. He said, "Don't worry, I will get you out of that fix," and began to ascend to a higher altitude. In a short time so many of the passengers had lost all notion of eating that there was a surplus of box lunches.

EARACHE

Changes in atmospheric pressure naturally affect any gas-filled cavity which is closed. Of chief importance in this regard is the middle ear. Normally, the Eustachian canal is only a potential tube, and it may be necessary to chew gum, yawn, and manipulate the tissues anterior to the head of the mandible in order to open the tube temporarily. If there is swelling due to a cold, the individual may suffer acutely, and sometimes it has been necessary to delay a landing because of this.

CONCLUSION

In conclusion it may be emphasized that many problems pertaining to aviation medicine await solution. Only a few have been mentioned here and notable omissions include pilot fatigue, cold, glare, vibration, etc., and the many problems of medical organization.

PHYSIOLOGY OF FATIGUE*

Factors and Criteria of Endurance

Lieut. Col. D. Bruce Dill, Dayton, Ohio

THE subject that I am going to discuss first is the dependence of fatigue on stresses. It is well-known to all of you in your experience as medical students that fatigue is a phenomenon that can be studied in an isolated muscle. The muscle can be given a task to do which at first it is able to do, and for some time, minutes or longer, it will continue to do at a rate which is maintained constant. Eventually a time will come when the work out of that isolated muscle will begin to fall off. That very simple conception of fatigue is applicable in some respects to all forms of fatigue, whether it is physical or mental. We say that fatigue is a state in which the organism has lost its capacity to carry on. It has a limited capacity for work. In the isolated muscle we can tell pretty well what some of these changes are. If the work has been carried on, for example, in an atmosphere of nitrogen, chemical changes can be detected by analysis which depend on the use of the energy reserves of that particular system anaerobically. If the system has been in an atmosphere of oxygen, work will be carried on longer, and the nature of

*Lecture XIX, reprinted from *Collected Lectures* of the Metropolitan State Hospital, Waltham, Mass., 1942.

the breakdown will be different. In the intact organism the picture is more complicated, but we can produce a state in the sprinter, for example, similar to the state seen in the isolated muscle. The sprinter is called upon to transform an enormous quantity of energy within a very short time. He has supplied nearly all of the energy from certain reserves that can be used without oxygen. When the work is done, there is a period of recovery in which extra oxygen is used, and eventually the original state of the organism is restored.

Another type of breakdown is one in which there is less heavy stress, but one of much longer duration. To give an illustration we enter another field of sport, that of the marathon race. In this we see a man working at high capacity for two and one-half or three hours. The chemical picture in this case is a very different one. The quantity of anaerobic energy available to a marathon runner is not of any significance. Since his energy expenditure is distributed over a period of two and one-half or three hours, he must rely upon oxidative processes. If we draw blood from a marathon runner when he has finished the race and compare its composition with that drawn from the man who has finished a sprint, we get important clues to the processes which have gone on within the muscle in the two cases. In the sprint we may find a blood sugar concentration of 150 or 200 mg. per cent. We may find a lactic acid concentration of 150 mg. per cent, which is to say fifteen times the normal resting level. The high blood sugar concentration in the sprinter may be interpreted as meaning that he has a copious supply of available carbohydrate. Because of the physical exertion plus the emotional factors involved, his reserve of carbohydrate has been mobilized and made available for this strenuous exertion.

In the marathon runner at the end of his race we may find blood sugar concentrations in occasional instances as low as 50 or 60 mg. per cent and lactic acid concentrations of 15 or 20 mg. per cent. However much the emotional situation may have raised the blood sugar in the beginning, by the time the race is over the carbohydrate reserves are depleted, blood sugar is apt to be at a low level, and the fuel is almost exclusively fat. The fact that his lactic acid concentration is low is evidence that he has not been expending energy anaerobically; oxygen has been supplied as fast as the fuels require it. These two contrasting conditions, the one depending largely upon anaerobic work, the other depending exclusively on aerobic work, represent two types of stresses placed upon the organism. The first is a very intense stress of short duration; the second, a less intense stress of longer duration.

The third type of stress to which attention must be paid is that of moderate degrees of exertion carried on for a very much longer time. We can give as an illustration of that the present-day laborer in heavy industries, for example, who still has hard work to do and who today may be working overtime—ten hours a day instead of seven. His fatigue is more like that of the marathon runner than it is like that of the sprinter; however, the biochemist cannot tell us very much about the fatigue of this laborer who may work ten or twelve hours a day. We know this man experiences fatigue, but it is composite in nature, made up in part of physical factors and in part of psychological, or if you wish, mental factors.

In that connection, I think it is worth while to refer here to the experience in England during the last year with overtime labor. This subject was studied very thoroughly in England during the last war, and the conclusion was reached that beyond some reasonable limit, nothing was gained by lengthening the work week. However, history repeats itself, and a year ago at the time of the collapse of the French, the British workman was called upon to give up his holiday weekend, to give up his leave, and to put in extra hours, mounting up to seventy or eighty hours a week. For a few weeks the output increased, and then it began falling off, and before long the weekly output had decreased. An inquiry was made by officials appointed to consider this particular question. They reached a number of significant conclusions; one of the most important is that under their conditions the most effective work week did not exceed fifty-five or fifty-six hours. They analyzed many individual instances of failure to maintain weekly output. After men had been tired out by these long hours, they decided they must have a day off. Instead of Sunday, when they received extra pay, they would take Wednesday. Among those who tried to put in these long work weeks there were more illnesses, increased loss of time because of accidents, and a general breakdown of morale. After this survey had been made, it was recommended that the work week should not exceed fifty-five or fifty-six hours. This experience has a practical lesson for America, because increasing pressure is being put on workmen for greater output. They are being encouraged to give up their vacations, and I understand that many workmen in this country are, at present, putting in seventy or eighty hours a week. This is a very shortsighted policy bound to fail of its purpose in the long run.

Another type of stress involves very little in the way of physical effort but does involve mental or emotional stress of various sorts. I am not qualified to discuss this here; it is a field in which you are experts and I am not, but I again want to give you a simple illustration, and perhaps at the end some of you will wish to make some comment on it. It is not only workmen who are called on to put in overtime; executives are doing so habitually. I think this is a mistake, whether in private business, among civil servants, or in military circles. The idea that a man can work at his desk for eight hours a day, go home and work another four hours at night is fundamentally unsound. It produces a fatigue that results in decreased output. Such an executive is likely to find himself going through the motions without thought or considerate action. I am going to risk saying that a good many of our difficulties today arise from the fact that the men at the top are too busy to think.

The second question which I wish to discuss is the dependence of fatigue on endurance. No matter what training is given a group of candidates for a track team, some will turn out to be superior and some inferior. In other words, one aspect of the dependence of fatigue on endurance is the native endowment. Whether we speak of the purely physical endowment or of mental characteristics, there are inherent differences which must be taken into account. Differences in native endowment are important in the selection of pilots as those of you who are medical officers well know. The same is true for selection of men in any walk of life. Our sprinter is a man with a very unusual

endowment; unusual mental powers are not required, but he does have legs of a particular type, a heart of unusual capacity, and a will to "put out." In prolonged physical effort a different type of endowment is required. The sprinter is likely to be a short man with heavy legs, whereas the successful marathon runner is commonly thin and wiry. We have made some tests of the capacity of various types of athletes in the Fatigue Laboratory. The one striking characteristic of the highly successful distance runner is his very great capacity for supplying oxygen to his body. The most notable example is Don Lash, the two-mile record holder of a few years back. He weighs 140 pounds or something of that sort. In a laboratory test we found that he could supply 5.35 liters of oxygen per minute. Most of us here cannot supply much more than one-half that much. That characteristic depends first on the capacity of the lungs to absorb the oxygen, of the heart to circulate the necessarily large quantity of blood required, and finally, a distribution of capillaries in tissues that favors the rapid delivery of as much oxygen to the cells that are doing the work.

The executive who can do this job of working twelve hours a day without breaking down must have very unusual mental endowment. He is a man who is able to take these continued stresses and bear up under them. He is able to consider one question at a time, settle it, drop it from his mind, and take up another. I say again that I am not qualified to discuss fatigue of this sort, but it intrigues me a great deal.

The other point important to this subject, i.e., to the dependence of fatigue on endurance, is, of course, the role of training. I said that any group of candidates for a track team could be trained indefinitely and great differences in performances would persist. However, all of these men are going to improve, and it is a very interesting physiological problem to determine the nature of this improvement. Why is it that a man can report for track team at the beginning of the year able to run a mile in five and one-half minutes and after three months' training become able to run one mile in four and three-fourths minutes? That improvement in the course of three months is not impossible. We have made a good deal of study of that development and know a good deal now as to just what happens in such an individual. In the first place, the improvement is, to a considerable extent, dependent upon skill or coordination. The man at the beginning is not merely covering a linear distance of one mile, but at every stride he is raising his body much more than is necessary. With every lift of his body a certain amount of energy must be expended. If you study moving picture records of the skilled runner as compared with the unskilled runner, you will see that the skilled runner goes along with only a slight undulation of his body; a large portion of his energy expenditure pushes his body forward.

There is also the development of conditioned reflexes. The coach at the beginning emphasizes time after time the proper methods of running. At first the runner takes thought for every stride, but eventually after he has done this for weeks or months he is able to run without giving thought to these aspects of his job, and then he has time available to consider the other aspects of running, to pay some attention to his competitors, and to judge the timing of his race.

That simple illustration applies to every walk of life. We have seen, for example, steel mill workers carrying out operations with ease that are simply impossible of attainment by the inexperienced man. That is possible because they have learned to do the job the easy way; they have acquired conditioned reflexes such that without giving thought to their job they can carry it through. That has great advantages in industrial jobs other than the mere conservation of physical energy. It means that the man who has learned his job well enough to carry it through without much thought has his mind free for reveries.

No doubt part of the role of physical training is the increase of muscle mass. The blacksmith has the large and powerful arm; it has become so in part because of the training it has had. No doubt muscles increase in size and strength with continued use; they hypertrophy if you wish to use that word. We have heard of the hypertrophy of the heart due to hard muscular effort over a long period of time. As far as I know, cardiologists have found nothing necessarily abnormal about large hearts in athletes.

In a man undergoing strenuous training there may be an increase in capacity for utilizing energy aerobically. A man before he has become trained may utilize three liters of oxygen a minute, and maybe 3.5 liters a minute after strenuous training. With prolonged and very strenuous training, it can be increased more than that. There may also be, as reported by Robinson and Harmon of the University of Indiana, an increased capacity for anaerobic utilization of energy. The highly trained man can push his blood lactate to twice as high a level as is attained by the untrained individual. That is an indication the anaerobic utilization of energy can be developed by long strenuous training.

The third topic I wish to discuss is the question of tests of physical fitness and the criteria of physical fitness. Unfortunately, there isn't any adequate, simple test of physical fitness. As physicians you all employ some simple test of physical fitness. You have your patients walk up and down stairs or step up on a chair or something of that sort, and you get some sort of idea as to their physical fitness. Some of you are familiar with Schneider's test. While useful for some purposes, this apparently doesn't give a reliable index to physical fitness. It is still used by flight surgeons, and it is believed to be useful in judging neurocirculatory stability. A reliable measure of physical fitness must put the whole body under a stress, calling for maximum effort, not merely by a small group of skeletal muscles but by the heart itself. Enough skeletal muscles must be put in use to approach the capacity of your cardiovascular system.

In one such test developed at the Fatigue Laboratory, the man first walks on a motor-driven treadmill at about 3½ miles an hour and on a 9 per cent grade. This is continued for about ten minutes, while we collect expired air, determine his oxygen consumption, measure his heart rate, make various blood tests, etc. This serves as a warming up period for the later test. After a few minutes' rest we speed up the treadmill to 7 miles per hour and have him run for five minutes or for a shorter time if that is impossible. In this test a measure of capacity of the individual to supply oxygen to his tissues is obtained;

this, to our minds, gives the most precise index of the over-all fitness of the cardiovascular system, and for that matter of the whole body as a machine. That figure, the maximum intake of oxygen, shows a wide variation among individuals, depending upon the original physical endowment, the training which a man has had, and the age of the individual. He reaches his maximum around the age of 20 to 25, and thereafter declines, perhaps as much as 50 per cent by the time he is 70. This sort of test is like that the engineer makes if he is called upon to compare the performance of two engines. He doesn't merely measure energy output and gas consumption while the engines are idling.

Some such test, if simplified, may have practical applications. We know that the Germans segregate unusually qualified individuals; very extensive psychological and neurological tests are made of men to find men of unusual mental capacities, and we know that they have troops sorted out on the basis of physical endowments. Storm troops were organized in the last war, and they are certainly playing a role in the present war. How can you select such men? That is a subject to which much thought has been given at the Fatigue Laboratory, Harvard University, during recent months. The "Pack Test," developed there and widely applied in both civilian and military circles, appears to give the most reliable index to physical fitness. Men who pass this test successfully not only are good athletes, they are tough fighters.

THE INFLUENCE OF AMPHETAMINE (BENZEDRINE) SULFATE AND CAFFEINE ON THE PERFORMANCE OF RAPIDLY EXHAUSTING WORK BY UNTRAINED SUBJECTS*

E. E. FOLTZ, M.S., M.D., M. J. SCHIFFRIN, M.S., PH.D., AND
A. C. IVY, PH.D., M.D., CHICAGO, ILL.

IT HAS been reported[1-3] that amphetamine sulfate, d-desoxyephedrine hydrochloride and caffeine increase endurance to muscular work of the type that is performed by trained subjects during a hike or a march with a pack. Caffeine has been shown to increase the rate of recovery in trained subjects after an exhausting ride on the bicycle ergometer.[3] We desired to ascertain the effect of amphetamine and caffeine, given one hour before the work period, on the output of work in *untrained* male subjects doing rapidly exhausting work.

METHOD

Twenty-three untrained male subjects were used. They reported to the laboratory one hour before each first work period to take their capsules. These contained a placebo, or 10 mg. of amphetamine, or 0.5 Gm. of caffeine sodium benzoate and were indistinguishable from one another.

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.
Aided by the Abbott Fund of Northwestern University.

The work was done by having each subject step up on a 16 inch wooden stand with one leg and down on the same leg once every three seconds until he could no longer maintain this rate. Each subject carried a knapsack on his back which was loaded, to the nearest 5 pounds, with a weight equal to one-third of his own weight. Each subject would work to fatigue, rest for three minutes on a near-by bed, then work to fatigue again. This procedure was carried out three times on each subject with at least a day's rest in between. One time the subject received the placebo; one time he received the amphetamine; and one time he received the caffeine. The subjects were staggered so that at the end of each double work period approximately one-third of them had received the placebo, another one-third the amphetamine, and the other one-third the caffeine.

SUMMARY OF WORK EXPERIMENTS

(23 Subjects)

TABLE I

EFFECT OF DRUGS

	1ST WORK PERIOD		2ND WORK PERIOD		TOTAL WORKING TIME		% RECOVERY	
	RANGE	AVERAGE TIME	RANGE	AVERAGE TIME	RANGE	AVERAGE TIME	RANGE	AVERAGE
	seconds		seconds		seconds		seconds	
Placebo	60-371	189	55-304	107	124-629	296	14-95	61
Amphetamine	83-513	203	46-305	112	134-818	315	20-96	60
Caffeine	56-520	181	42-272	104	102-765	285	33-86	61

TABLE II

EFFECT OF TRAINING

	1ST WORK PERIOD		2ND WORK PERIOD		TOTAL WORKING TIME		% RECOVERY	
	RANGE	AVERAGE TIME	RANGE	AVERAGE TIME	RANGE	AVERAGE TIME	RANGE	AVERAGE
	seconds		seconds		seconds		seconds	
Experiment 1	56-251	142	42-184	84	102-411	226	20-91	63
Experiment 2	60-361	166	55-201	96	115-629	262	38-95	61
Experiment 3	102-520	265	69-305	144	178-818	409	30-96	59

RESULTS

The results are to be found in Tables I and II. In Table I are the data showing the results with the placebo, amphetamine, and caffeine. As is evident there is no significant difference between the three. In Table II are the data showing that the subject's work output improved with practice. It is evident, then, that training had a much greater effect on performance than did the drugs. It will be noted that the general per cent recovery remains very constant in both tables. Table II, however, shows a tendency for it to decrease as the work output of the first period increases, which is in keeping with what we have found to be true in the past.[3]

The drugs produced no side effects, except that they caused most of the subjects to report that they felt euphoric and as if they could do a "lot of work today." The euphoria, however, did not permit them actually to do more work of the type performed.

CONCLUSIONS

1. In this experiment the effect of training overshadowed any effect the drugs may have exerted. This type of test is inadequate to determine the effect of the drugs used on work output. The *experiment emphasizes the importance of training.*

2. The average per cent of recovery in this series is very constant.

3. The average per cent of recovery tends to decrease as the average work output of the first period increases.

REFERENCES

1. Ivy, A. C., and Krasno, L. R.: Amphetamine (Benzedrine) Sulfate; Review of Its Pharmacology, War Med. 1: 15, 1941.
2. Ivy, A. C., and Götzl, F. R.: d-Desoxyephedrine (Pervitin or Methedrine), War Med. In press.
3. Foltz, E. E., Ivy, A. C., and Barborka, C. J.: The Use of Double Work Periods in the Study of Fatigue and the Influence of Caffeine on Recovery, Am. J. Physiol. 136: 79, 1942.

THE INFLUENCE OF AMPHETAMINE (BENZEDRINE) SULFATE, d-DESOXYEPHEDRINE HYDROCHLORIDE (PERVITIN), AND CAFFEINE UPON WORK OUTPUT AND RECOVERY WHEN RAPIDLY EXHAUSTING WORK IS DONE BY TRAINED SUBJECTS*

E. E. FOLTZ, M.S., M.D., A. C. IVY, PH.D., M.D., AND C. J. BARBORKA, M.S., M.D., D.SC., CHICAGO, ILL.

INTRODUCTION

SINCE it was found by Foltz, Schiffrin, and Ivy[1] that practice or training overshadowed any effects that amphetamine and caffeine may have exerted on the work output of untrained subjects doing rapidly exhausting work, this study on trained subjects was undertaken. It has been shown that caffeine increases the rate of recovery from exhausting exercise in trained subjects.[2]

METHODS

Four medical students were used. They were provided with board and room in a hospital near the laboratory. Their food intake was under control; their diet was adequate in all known components, a certain variation in the selection of food items being permitted. The subjects were chosen on the basis of their willingness to cooperate, and not on the basis of muscular development, the work of the tests being their only source of physical exercise. The double work periods occurred on Monday, Wednesday, and Friday afternoons at the same time between 2 and 4 o'clock. The subjects worked on the bicycle ergometer described by Kelso and Hellebrandt,[3] at a rate of 1,235 kilogram meters (kg.m.) per minute, with a pedaling rate of 54 r.p.m.

*From the Departments of Medicine, and Physiology and Pharmacology, Northwestern University Medical School, Chicago.
Aided by the Abbott Fund of Northwestern University.

The periods of training of the subjects prior to performing these tests varied from three to sixteen months. The subjects worked to complete fatigue, rested ten minutes, and then worked to complete fatigue again.[2] The end point of fatigue was the point at which they could no longer hold the needle on the dial at the rate of 54 r.p.m.

The amphetamine (10 to 15 mg.), desoxyephedrine (5 mg.), and caffeine sodium benzoate (0.5 Gm.) were always administered intravenously. They were given at various times (thirty seconds to 30 minutes) prior to the first work period to determine their effect upon work output, and amphetamine and desoxyephedrine were given immediately after the first work period to determine their effect upon recovery, as measured here, from fatigue. The effect of caffeine upon recovery is reported elsewhere.[2] Placebo injections (sodium chloride solution) were given at random as controls. All solutions were colorless. The drugs were given intravenously to obtain rapid action, and because this group of subjects had previously obtained various therapeutic agents by a similar route to control the rate of absorption.

RESULTS

Various subjective sensations were reported by the subjects. After the injections of amphetamine, two subjects felt definitely "keyed up," and both experienced more leg pain during and after the work periods. The other two subjects reacted identically to both placebo and amphetamine. Desoxyephedrine seemed to increase the leg pain in one, to decrease it in another, and to cause insomnia in three subjects. In one case, when it was given immediately after the first period, it seemed to dispel fatigue. This sensation of being rested would last until he had worked for about ten seconds in the second period, when he would begin to feel as tired as though he had only had a placebo injection, and his recovery was not enhanced. This is in contrast to the response of this subject to 0.5 Gm. of caffeine sodium benzoate, given in the same manner.[2] This drug would also stimulate the subject, but the stimulation would last throughout the second period, and greatly increase his recovery. After being given either desoxyephedrine or caffeine for the rest of the day, this subject would feel extremely alert, and would experience difficulty going to sleep at night. One subject complained of weakness and giddiness following the first work period, after having received desoxyephedrine prior to working, and one reported no sensations from the desoxyephedrine. All four subjects reported definitely stronger immediate subjective sensations from the caffeine and desoxyephedrine when the drugs were given before the first period than when they were given after the first.

The results obtained by giving the drug before the first period are shown in Table I. It is evident that the amphetamine had no significant effect. However, desoxyephedrine and caffeine definitely increased the work output. With the increase of work done in the first period, the expected decrease in per cent recovery occurred.[1]

The results obtained by giving the drug immediately after the first period are shown in Table II. In the case of amphetamine it appears that the work output was enhanced. However, if this were true, the increment would

be in the second period work output and the per cent recovery would be higher than that of the controls. Because these qualifications are not fulfilled, no effect can be attributed to amphetamine. In the case of desoxyephedrine the per cent recovery was increased over that of the controls. However, if this were significant, certain other data should be present; namely, the first period work output of both the desoxyephedrine and the control series would be approximately the same, or that of the desoxyephedrine would be more; the second period and total work outputs would be significantly increased in the case of the desoxyephedrine. Since such is not the case, it cannot be said that desoxyephedrine significantly increased recovery.

TABLE I

DRUG GIVEN BEFORE THE FIRST PERIOD

SUBSTANCE	TRIALS	SUBJECTS	DOSE IN MG.	1ST PERIOD WORK OUTPUT (KG.M.)	2ND PERIOD WORK OUTPUT (KG.M.)	TOTAL WORK OUTPUT (KG.M.)	% RECOVERY
Amphetamine	10	2	10-15	4,570	2,862	7,432	63
Control†	10	2	----	4,793	3,117	7,910	66
Desoxyephedrine	12	4	5	8,870	5,750	14,620	66
Control†	12	4	–	7,055	5,105	12,160	72
Caffeine	6	4	500	5,805	3,911	9,716	66
Control†	6	4	–	5,201	3,667	8,868	70

TABLE II

DRUG GIVEN IMMEDIATELY AFTER THE FIRST PERIOD

SUBSTANCE	TRIALS	SUBJECTS	DOSE IN MG.	1ST PERIOD WORK OUTPUT (KG.M.)	2ND PERIOD WORK OUTPUT (KG.M.)	TOTAL WORK OUTPUT (KG.M.)	% RECOVERY
Amphetamine	17	4	10	6,764	4,167	10,931	63
Control†	17	4	--	6,478	3,931	10,409	62
Desoxyephedrine	31	4	5*	6,660	4,692	11,352	70
Control†	31	4	–	6,790	4,510	11,300	66

*In six trials less was used.

†Variations in the work outputs of the several control series result from different stages of training.

SUMMARY

The effect of amphetamine, desoxyephedrine, and caffeine upon work output, and the effect of amphetamine and desoxyephedrine upon recovery in subjects doing rapidly exhausting work have been observed. All three drugs stimulated some of the subjects mentally, so that they felt "keyed up" and more alert. The immediate subjective sensations resulting from desoxyephedrine and caffeine were much more pronounced when the drugs were given to a nonfatigued subject than when given to a subject following exhaustion. Amphetamine did not enable the subjects to work longer, nor to recover more rapidly. Desoxyephedrine did enable the subjects to work longer, when it was given before work began, but it did not hasten recovery when given to the exhausted subjects. Caffeine also enabled the subjects to work longer and, in addition, hastens recovery when given to exhausted subjects.[2]

CONCLUSIONS

As measured by this method:

1. Ten to 15 mg. of amphetamine injected intravenously do not increase work output in the trained subject doing rapidly exhaustive work.

2. Five milligrams of desoxyephedrine injected intravenously into the nonfatigued subject increase work output.

3. One-half gram of caffeine sodium benzoate injected intravenously into the nonfatigued subject increases work output.

4. Neither 10 mg. of amphetamine nor 5 mg. of desoxyephedrine injected intravenously into the fatigued subject actually enhance the rate of recovery from heavy work carried to the point of "exhaustion"; caffeine in adequate doses does.[2]

5. When stimulants are employed, they should be chosen according to whether it is desired to increase the work output of unfatigued subjects, or to enhance the recovery of fatigued subjects.

The drugs in some instances definitely improve subjective feeling tone and cause the subject to feel as though he can and is actually doing more work.

It should be remembered that the foregoing observations pertain only to rapidly exhausting work, and not to the type of work performed during a march or while standing guard.

REFERENCES

1. Foltz, E. E., Schiffrin, M. J., and Ivy, A. C.: The Influence of Amphetamine Sulfate and Caffeine on the Performance of Rapidly Exhausting Work by Untrained Subjects, J. LAB. & CLIN. MED. 28: 601, 1943.
2. Foltz, E. E., Ivy, A. C., and Barborka, C. J.: The Use of Double Work Periods in the Study of Fatigue and the Influence of Caffeine on Recovery, Am. J. Physiol. 136: 79, 1942.
3. Kelso, L. E. A., and Hellebrandt, T. A.: Recording Electrodynamic Brake Bicycle Ergometer, J. LAB. & CLIN. MED. 19: 1105, 1934.

PLANTAR REFLEX AS A CRITERION OF ENDURANCE*

PAUL I. YAKOVLEV, M.D., WALTHAM, MASS.

YOU HAVE just heard an authoritative and instructive discussion of the problem of fatigue and endurance by Dr. Dill. I am quite aware, therefore, that I am taking a measure of liberty in adding a few remarks of my own on the behavior of the plantar reflex following a prolonged locomotion as a criterion of endurance.

It is generally known that the Babinski sign or plantar extensor response of the big toe occurs as a result of a deficiency of the function of the corticospinal tract. It is usually taken as a sign of a grave disease; in fact, of a destructive lesion in the nervous system. Such a view, however, is incorrect. The extensor response of the big toe is normal in infants up to eighteen months of age. It is also elicitable in normal adults during a profound sleep. It is evident, therefore, that a Babinski sign need not imply necessarily a structural damage to the corticospinal tract, for it may occur in normal physiologic states. However, in individuals with a constitutional weakness or fragility of the nerv-

*Lecture XX, reprinted from *Collected Lectures* of the Seventh Postgraduate Seminar in Neurology and Psychiatry, first semester, Military Neuro-psychiatry, Metropolitan State Hospital, Waltham, Mass., 1942.

ous system, the Babinski sign occurs readily under varying conditions which ordinarily are insufficient to cause its appearance in the constitutionally well-endowed persons. It may occur, for example, after a prolonged exertion, such as a long march. I would like to make clear that potentially a Babinski sign may well develop in any individual if the amount of exertion and fatigue is brought to the point of severe exhaustion, or beyond that point. However, in the constitutionally inferior individuals it develops after a relatively small amount of exertion, insufficient to cause its appearance in normal people. The occurrence of a Babinski sign after an ordinary amount of exertion in a person otherwise appearing healthy is a cogent indicator of a constitutional inferiority of such a person, and may serve as a criterion of endurance. As a test of endurance it has the advantage of being technically simple.

The assertion of the significance of the "exertion Babinski" as a criterion of endurance is based on the following observations:

Three years ago Farrell and I[1] undertook to study the effect of exertion, and particularly of a prolonged locomotion on the plantar reflex in a group of young and healthy college graduates in a reserve officers' training camp, and in a group of the constitutionally inferior persons at a farm colony for mental defectives connected with Walter E. Fernald State School. The results were as follows:

In a group of 168 college graduates in a reserve officers' training camp the plantar reflex was examined before and after a march of fourteen miles during military maneuvers. Of the 168 men thus examined, a Babinski sign developed after the march in 12, or 7.2 per cent. In 10 of these young and otherwise healthy men, a Babinski sign developed unilaterally and in 2 bilaterally. Having thus obtained a relative measure of the effect of exertion upon the plantar reflex in a group of normal individuals, we have examined the plantar reflex before and after seven hours of ordinary farm work in a group of 229 adult imbeciles at the state colony. Of 229 mental defectives thus examined, a Babinski sign developed in 49, or 21.4 per cent. The incidence of exertion Babinski after a day of ordinary physical work in this group of constitutionally inferior persons was, therefore, three times that of its incidence in a group of college graduates after fourteen miles of forced march.

Effect of locomotion. When a group of 60 mental defectives selected among those in whom after a day of ordinary farm work the Babinski sign did not develop were put to a test of a march of ten miles, 13 per cent of them developed a Babinski sign. After a march of an additional fourteen miles, 23 per cent developed a Babinski sign. It should be evident that locomotion was a particularly effective mode of exertion, causing the Babinski sign to appear in certain predisposed individuals.

The incidence of an "exertion Babinski" is as high in individuals presenting other evidence of constitutional inferiority. Thus, last summer Semrad and I[2] examined the plantar reflex in 64 adult males with various forms of chronic psychoses, chiefly schizophrenia, before and after a walk of only six miles. After the walk 10 per cent developed a frank Babinski sign unilaterally or bilaterally.

When the factors underlying the occurrence of "exertion Babinski" were investigated closer, interesting facts came to light. It was found that the individuals who endured a prolonged locomotion *without developing a Babinski sign* showed a relatively normal time of development of locomotion in infancy. They walked at about the normal age of *one and a half years.* On the contrary, the individuals *who developed a Babinski sign* after exertion began to walk much later, namely, at an average of *two and a half years of age,* and those of them *who developed a bilateral Babinski sign* were the most delayed in the age of establishment of erect locomotion, for they learned to walk on the average as late as at *three and a half years of age.*

It should be quite evident, therefore, that in certain individuals the occurrence of Babinski sign under influence of exertion stands in direct relation to their constitutional endowment. It appears to be a pertinent indicator of the constitutional stamina of the individual. I believe this fact is of a distinct practical interest for military medicine. A soldier who after a march of a few miles or at the end of a day of routine work develops an extensor response, especially a bilateral Babinski sign, regardless of his intellectual and physical status otherwise, should be considered a poor choice for an assignment to special task units in which physical endurance is a prime requirement, although this limitation does not preclude such a soldier to be proficient in other military services.

Concluding, I would like to emphasize that the occurrence of "exertion Babinski" has nothing to do with the "intelligence" of the individual. It may occur in mentally normal and physically healthy college graduates as well as in imbeciles and in psychotic individuals, although in the latter groups it occurs more readily and more generally than in the former group.

REFERENCES

1. Yakovlev, Paul I., and Farrell, Malcolm J.: Influence of Locomotion on the Plantar Reflex in Normal and in Physically and Mentally Inferior Persons, Arch. Neurol. & Psychiat. 46: 322-330, 1941.
2. Yakovlev, Paul I., and Semrad, Elvin V.: Unpublished data.

MILITARY ASPECTS OF ALLERGY*

CAPTAIN PHILIP BLANK, M.C., U. S. ARMY

THE advent of drafting men into the armed forces has brought with it the problem of allergy to confront the Army Medical Corps. Until now, problems involving allergic diseases have been minimized by the simple expedient of refusing to admit into the service all men with a history of a major allergy, and by discharging from the service those persons whose allergic problems became too troublesome. However, man power is important; it must be conserved and it must be utilized as advantageously as possible. The drafting of class IB men increases the problem of the conservation of man power, and the allergist is participating in the solution of this problem by aiding in the treatment and assignment of these physically inferior men to places and duties that will utilize to the greatest extent possible their abilities, and at the same time release for combat duty those men who are physically able to do the more arduous work.

In World War I the specialty of allergy was too new to attempt the solution of so vast a problem as was presented at that time. Allergy then had no official recognition, nor is it recognized officially by the Army at present. The result is that we possess rather poor statistics for most allergic diseases treated during the period previous to the present war. Fairly adequate statistics on asthma alone are available in Volume 15 of the statistical report of the Army Medical Department.[1]

During the last war the draft board rejections for asthma with emphysema and bronchitis averaged 2.45 per 1,000.[2] It should be noted, however, that not all asthmatics were included in this category, since associated emphysema or bronchitis was necessary for rejection. In the Army between April, 1917, and November, 1919, approximately 2.62 white soldiers per 1,000 and 5.60 colored soldiers per 1,000 were admitted to hospitals with the diagnosis of asthma; and 2,948 soldiers were discharged for disability arising from that defect. This represents 5 men per 100,000. Asthmatic cases caused the loss of 190,273 days. During World War I only 2 men per 1,000 were diagnosed as asthmatic, whereas fifteen years later 12 per 1,000 were receiving compensation for this ailment.

Since World War I the field of allergy has grown to such a proportion that it is now capable of dealing with questions of allergy as a distinct military problem. Thus, in place of the inadequate treatment of the past, allergic diseases are now diagnosed and adequate treatment is attempted. Reclassification is substituted for over-all discharges, and conclusions based on inadequate information are replaced by decisions having their foundation in known and proved data.

---

*From the Station Hospital, Fort Eustis, Virginia.

In World War II the latest statistics furnished by the office of the Surgeon General[3] indicate an annual admission rate of 2.57 per thousand per annum for allergic disease, the certificate of disability discharge rate is 0.40 per thousand per annum, and the death rate is nil.

From these statistics of World War I and II it is evident that the problem of allergy is not a small one and, considering its nature, very difficult.

The military aspect of allergy resolves itself into three separate problems. The first concerns itself with the professional duties of the Army allergist; the second with the correct distribution of allergists in the tables of organization of the medical department; and the third with the formulation of specific physical standards for all major allergic disorders.

The professional duties of the Army allergist deal with the diagnosis and prognosis of allergic diseases. A discussion of the routine methods of diagnosis used in the field of allergy does not fall properly within the realm covered by this dissertation. However, the prognosis of the ability of the allergic soldier must be considered, and it must be emphasized that this is the most important single task of the Army allergist. He, while attempting to perform this task successfully, must discover the answers to numerous and perplexing questions. Is the soldier fit for duty or should he be rejected or discharged? If fit for duty, does this mean full duty or limited service? If the conclusion is that the man is suited for limited service only, what are the services he is capable of performing and where can he perform them to the best of his abilities?

Not only is the allergist capable of handling the major allergies, such as asthma and hay fever, but his services are of indispensable value to the dermatologic, eye, ear, nose, and throat sections of his hospital. It is he who should investigate and control the contact skin problems, especially those cases involving poison ivy and "laundry dermatitis." Furthermore, many cases seen in these departments will respond promptly to an allergic regime but not to ordinary methods of treatment.

Inasmuch as the amount of serum and vaccine therapy is extremely great in the Army, the supervision of these therapeutic measures is a duty which should fall in the realm of the allergist. It is well known that all soldiers receive typhoid, smallpox, and tetanus prophylactic measures. If indicated, yellow fever, typhus, and cholera prophylaxis are administered. Methods of administration and control of reactions require constant supervision in order to minimize untoward reactions.

It is obvious that the correct distribution of allergists in the Army is of prime importance in the conservation of the material and men needed to carry on this type of work. Since the most important work of the allergist is concerned with the prognosis of a soldier's ability, it becomes evident that the allergist is best able to perform his duties where troops are inducted and trained. However, under the present organization of induction and reception centers, little time is available to the allergist to permit him to do more than eliminate those men who have no military value. It is rather at the training centers that the soldiers first come into contact with actual Army activities for

a period sufficient in duration so that they may be studied, their symptoms may be evaluated, and disposition of their cases may be made. Large general hospitals should also have allergists to take care of the allergic diseases discovered in the forward echelons.

The problem of allergy in the Army is further complicated because as yet adequate standards covering the allergic diseases have not been formulated by the Army Medical Department. This does not necessarily reflect adversely on the medical branch of the Army, but rather on the newness of the field of allergy. The formulation of an adequate allergy department must proceed slowly and must await the results and statistics uncovered by those medical officers sincerely interested in this field.

From a communication of the Surgeon General's[3] office regarding the physical requirements pertaining to the person suffering from an allergic disease, the following is taken:

"The physical standards for admission to the Army make no reference to specific allergic conditions other than hay fever. Individuals with mild hay fever are accepted for general military service, those who have moderate hay fever are accepted for limited military service, and those who have severe hay fever are rejected. Other important allergic conditions may be disposed of as indicated in Paragraph 5 *a* MR 1-9, revised October 15, 1942, which reads as follows:

'If any individual is regarded by the medical examiner as physically unfit for military service by reason of physical or mental defects not specifically noted in these regulations, he will nevertheless be recommended as unsuitable for general service or for limited service as the case may be. A brief statement of the reasons for the rejections will be entered on the report of physical examinations. So far as practicable, however, the physical classification of individuals will conform to the specified requirements.' "

To illustrate the problem of allergy in the Army, the statistics obtained at the allergy clinic at Fort Eustis, Virginia, from June 1, 1941, to October 1, 1942, are here presented and evaluated.

Table I represents the total number of cases seen at the allergy clinic and includes both outpatients referred to the clinic by the various infirmaries on the post and the hospitalized cases. No effort has been made to include in the tabulation those soldiers seen at the various infirmaries who were not sent to the clinic. The figures regarding the hospital cases were taken from the official diagnosis file kept by the registrar of the hospital and represent only those cases with the primary diagnosis as stated.

The total number of cases observed at the clinic represents approximately 1.5 per cent of all personnel at this post. It should be noted that this estimate is lower than is usually quoted; however, it is not a completely accurate reflection of the situation because undoubtedly many soldiers afflicted with minor allergies did not report to the infirmary or were not referred from the infirmary to the clinic. We may anticipate that over 1 per cent of all men entering the Army will require allergic care.

A perusal of the table indicates that the respiratory problems form the largest group among the allergic diseases; in fact, slightly over half of all allergic cases observed belong in this category. The greatest number of cases in this respiratory group are those of seasonal hay fever, these representing

slightly less than half of the total respiratory cases and 22 per cent of all allergic problems. The ragweed pollen cases far exceed those involving grass pollen; only a very few tree pollen cases have found their way to the allergy clinic. It is encouraging to realize that all the hay fever cases constitute a group for which excellent therapeutic results may be expected with only a moderate loss of man hours. The problem is still further simplified because there is a definite territorial ragweed belt. This belt is confined mainly to the areas of continental United States and Canada east of the Rocky Mountains (short and giant ragweeds), and thus all ragweed cases will be free from ragweed hay fever so long as they remain outside this area.

TABLE I

STATISTICS OF THE ALLERGY CLINIC, STATION HOSPITAL, FORT EUSTIS, VA. FROM JUNE 1, 1941, TO OCTOBER 1, 1942

TOTAL CASES OBSERVED	NUMBER OF CASES	% CASES	HOSPITAL CASES	C.D.D.* CASES	ADMISSION RATE§ PER 1,000	RATE OF C.D.D.* PER 1,000
	1,833	100%	380	78	15.0	0.60
Respiratory Allergies	919	50.1	152	75	7.52	0.56
Hay fever	417	22.7	4	0	3.41	0.00
Asthma	289	15.7	92	75	2.36	0.57
Perennial rhinitis	161	8.7	51	0	1.31	0.00
Sinusitis (allergic)	28	1.5	†	0	0.23	0.00
Nasal polyps	24	1.3	5	0	0.20	0.00
Dermatologic Allergies	773	42.1	175	3	6.32	0.03
Contact dermatitis	346	18.8	110	0	2.82	0.00
Id type reactions	128	6.9	†	0	1.04	0.00
Urticaria	127	6.9	16	1	1.04	0.01
Dermatitis medicamentosa	76	4.1	15	0	0.62	0.00
Atopic dermatitis	57	3.1	32	2	0.47	0.02
Angioneurotic edema	39	2.1	2	0	0.32	0.00
Ophthalmologic Allergies	130	7.0	49	0	1.05	0.00
Conjunctivitis	104	5.6	23	0	0.84	0.00
Ulcerative keratitis	26	1.4	26	‡	0.20	0.00
Gastrointestinal Allergies	5	0.2	1	0	0.03	0.00
Migraine (allergic)	6	0.2	3	0	0.03	0.00

*Certificate of disability discharge.
†Not classified according to this diagnosis.
‡One case transferred to Walter Reed General Hospital.
§Admission rate to clinic per 1,000 men on post.

The policy of this clinic regarding these cases has been to continue treatment of all persons who have previously received medical care for this disease. Many cases have been treated preseasonally or perennially prior to induction into the Army. The cases of mild severity have been treated symptomatically with eye drops, nose drops, or ephedrine by mouth as is indicated. The moderately severe and severe cases are treated coseasonally with small doses intradermally accompanied by symptomatic treatment. Almost all these soldiers were treated in an ambulatory fashion; only four cases were hospitalized. Those men who suffer severely were instructed to secure perennial treatment as long as they remain in the ragweed belt. The results of therapy have been exceedingly gratifying.

Although the asthmatic group represents only 15.7 per cent of all allergic cases observed, this group nevertheless poses the most difficult problems. It is here that the allergist must be extremely perspicacious in order to attain correct disposition of the case. It is our opinion that practically all asthmatics, except those of mild seasonal type, should be reclassified into limited service.

In our clinic we have divided our asthmatic patients into four classes: first, the seasonal asthmatics; second, the old chronic asthmatics with or without secondary complications who have never been capable of pursuing a civilian occupation without a great deal of lost time; third, mild asthmatics who have been able to accomplish useful work in civilian life without undue loss of time; and fourth, those asthmatics who have had their first attack while in the Army. The seasonal asthmatics are treated similarly to severe hay fever cases, and no doubt can do well in foreign service, especially with noncombatant units. Old chronic asthmatics with secondary complications should be discharged from the service as soon as the diagnosis is made. Therapeutic results with this class are very unsatisfactory under military conditions. Asthmatics who have never been successful in civilian employment can hardly be expected to be of any greater value in military service. Thus, it is extremely beneficial both to the Army and to these men that they be discharged as promptly as possible. Those asthmatics without complications who have been able to follow a vocation in civilian life without undue loss of time are reclassified for noncombatant duties. The positions to which they are assigned should simulate their civilian occupations as closely as possible. These men can be hyposensitized and a rather moderately effective allergic regime can be instituted because their reclassification into noncombatant units makes treatment possible. Territorially, these men can be restricted to specific areas.

The last class of soldiers suffering from asthma includes those persons whose cases in the asthmatic group are the most difficult to handle. These men have had no or few previous attacks. They generally possess a past history of some allergic manifestation and a familial history of allergy. These cases should be skin tested for those materials which they now encounter in their new Army environment. One must always keep in mind the high concentration of certain substances in the barracks, e.g., feathers, tobacco smoke, mattress, blanket, and wood dust. Experience at this clinic has indicated that barracks dust is much more antigenic than ordinary house dust, and we have attributed this condition to the above-mentioned factors. It has been found that many patients allergic to barracks dust respond promptly to a coseasonal type of treatment, i.e., small frequent doses intradermally. If such measures do not evoke rapid and proper response from these men, they should be reclassified. Trials of duty are essential in forming an opinion in these cases. If the patients become progressively worse, they should be discharged from the service.

The remaining cases in the respiratory group may be termed harassing diseases. Persons falling in this category are frequently on sick call or are attending the ear, nose, and throat clinic. Many of these cases can be controlled by the judicious use of ephedrine drops, others by the elimination of the offending inhalant or by hyposensitization to the antigenic substance.

Nasal and sinus polyps may require surgical removal. Nevertheless, they should be examined from an allergic aspect and, if feasible, measures should be taken to prevent their recurrence.

The second large group of cases with which the allergy clinic is confronted consists of the dermatologic allergies. As might be anticipated, the cases of contact dermatitis occupy the predominant position. In this group the most habitual offender is poison ivy, and the parts of the body usually affected are the hands, arms, legs, face, and penis. The most efficacious therapeutic measure which should be instituted is prophylaxis, which is extremely simple because it can be accomplished by the use of material available to all soldiers—G. I. soap. If persons who are aware of their susceptibility to plants, or who know or suspect they have contacted poison ivy, will merely bathe with G. I. soap as soon as possible after contact, making certain to wash the parts of the body most frequently affected, much of the dermatitis venenata due to poison ivy will be eliminated. It must be emphasized that overtreatment of these cases produces unfortunate results. The value of the injectable extract of poison ivy twenty-four to forty-eight hours after contact is questionable, and is too costly for routine use beforehand. Most posts will have fewer casualties from this disease if by cooperative efforts of the allergist and post medical inspector there is an intelligent mapping and clearing of areas known to have poison ivy.

Wool is another important irritant. To determine whether or not a patient is sensitive to this material, a patch test, consisting of a patch of the wool underwear or shirt the soldier has been wearing, is used. However, such a test does not eliminate "laundry dermatitis." As has been reported in a previous article by the author,[4] the majority of our so-called "wool dermatitis" cases have been caused by laundry soaps and more recently by waterproofing fluid. "Laundry dermatitis" occurs more often where thick fabrics are involved, because, if woolen and cotton articles are subjected to an equal amount of rinsing, a greater proportion of the laundry materials used in washing will remain in the heavier than in the lighter garments. By enlisting the aid of the laundry officer so as to insure more thorough rinsing, the so-called wool dermatitis cases drop remarkably.

Other objects giving rise to contact dermatitis are the aluminum in the identification disks worn by the soldiers, the metal parts of watches, and the plastics used as wrist bands. There was one case of ulcerative stomatitis which was due to sensitivity to dentures, the patient being sensitive to all types of dentures except vitallium.

Urticaria is probably much more frequent than the reported figures would indicate because of the great number of vaccine injections administered in the Army and the drastic transformation undergone by a man who changes his status from civilian to soldier. This is because most of the hives are quite evanescent. The majority of the cases of hives seen at the clinic are those that have either lasted several days or are of the persistent or recurrent type. Most of those in the nonpersistent group clear quickly, but those of the recurrent type offer a very difficult diagnostic problem inasmuch as many cases are not allergic in origin. Other factors must be sought, especially those of a neurogenic character. It is rather astounding to note how much a chaplain's per-

sonal conversation with a patient, when accompanied by a little sedation, can accomplish in the way of alleviating a case of hives on a functional basis.

Atopic dermatitis cases should be handled in much the same way as the asthmatics. If a case is mild, tar ointments and moderate control will result in marked improvement; if moderate, the patient should be reclassified. The latter cases require definite allergic regimes and therapy which can only be given in the rear echelons. The severe cases should be discharged from the service, and this should certainly be the solution adopted after a trial of therapy and a trial of duty result in little or no improvement. Throughout any discussion of allergy in the Army, it must be remembered that each soldier represents a financial investment to the Government, and if the return on the investment does not warrant the expenditure, the sooner the financial operation is terminated the better for all concerned. It must also be recognized that of all the diseases, except venereal, confronting the Army, the dermatologic ones can destroy the morale of soldiers more rapidly than any other group of diseases. Men are exceedingly sensitive to deformities and to skin disorders both in themselves and in others. Thus, it is often wise to transfer from combat branches to service units those persons possessing unsightly skins if the skin disorders are of a permanent or semipermanent nature. This will prevent the disintegration of morale among the fighting divisions of the Army where morale is especially vital.

Dermatophytids of the hands are quite common in the Army, but they should be differentiated from other lesions, such as nummular eczema, contact dermatitis, and psoriasis. However, once dermatophytids are recognized, they respond exceptionally well to local treatment of the original lesion accompanied by intradermal doses of the fungus extract for the purpose of hyposensitization.

Many examples of drug allergies are seen at the post and these are especially noticeable in the wards of the hospital. At present, the sulfone drugs cause the most trouble. Cases involving drugs rarely represent an allergic problem except as a matter of diagnosis, and the diagnostician is hindered by the lack of good methods of testing. It is hoped that the latter problem will soon be solved by a more effective testing procedure than is presently employed.

Although the ophthalmologic allergies represent only a small group of the allergic diseases observed at our clinic, they are nevertheless quite fascinating. It should be noted that we have reported separately only those cases in this group devoid of concomitant symptoms. Thus, where conjunctivitis has been accompanied by hay fever, rhinitis, or asthma, the statistics have been included under the specific headings of the respiratory allergies. The majority of the cases of conjunctivitis were due to pollens and dust. Conjunctivitis may also be classified as an harassing disease because it leads to much physical discomfiture and thus decreases the efficiency of the soldier. Most conjunctivitis cases respond well to hyposensitization, but overdosage must be carefully avoided as keratitis may result. We have found small intradermal doses, combined with local treatment, to be most satisfactory.

As we have previously reported,[4] an unusually large number of ulcerative keratitis cases have been observed at this clinic. The clinical picture is that of small superficial ulcers located around the circumference of the cornea about

1 or 2 mm. from the limbus. These ulcers are very small and shallow, and are usually multiple; with progression they increase in size and coalesce and may form a continuous marginal ulcer. Marked conjunctivitis accompanies the keratitis. While pain is not usually present, itching may be intense. Central keratitis sometimes occurs secondarily due to lack of nourishment. Most cases respond well to hyposensitization, but hospitalization is indicated.

The gastrointestinal cases and the other minor allergies constitute interesting diagnostic situations for the allergist, but they are not significant as a military problem because of the small number of cases involved.

The statistics of the Station Hospital at Fort Eustis, Virginia, reveal that 1,833 cases were observed at the allergy clinic. This represents an admission rate to the clinic of 15 men per thousand for allergic study, treatment, and disposition. Slightly over 20 per cent of these cases were admitted to the hospital, or about 3 men per thousand were hospitalized for allergic disease. This figure compares favorably with the admission rate quoted earlier from the office of the Surgeon General of 2.57 per thousand. It must be noted, however, that as only a small proportion of the cases of major allergy were admitted to the hospital, the problem is actually much larger than is indicated by the admission rates to the various hospitals.

The total number of men discharged from the Army at Fort Eustis as being unfit for any military duty is 78, which represents 4.3 per cent of all cases seen at the clinic, or 0.60 men per thousand on the post. This also compares favorably with the certificate of disability rate of 0.40 per thousand, as reported by the office of the Surgeon General.

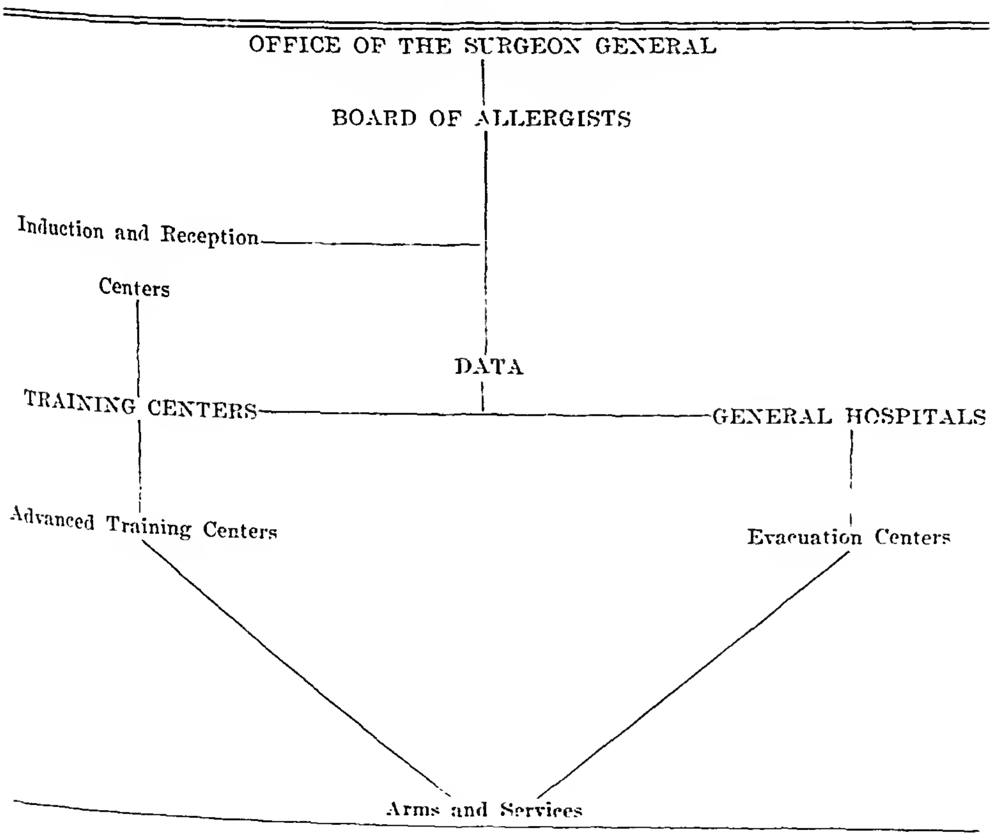
This dissertation is an attempt to present a comprehensive analysis of the military aspects of allergy in the Army. The study has led me to suggest the organization of the Army allergy service. I do not profess to possess the qualifications needed to set up the vast organization which would be required to take care of the thousands of men in the Army of the United States who suffer from allergic diseases, but I rather offer this suggestion with the hope that it will be of some constructive assistance in the solving of the problems of allergy in the armed forces.

The training camp is the focal point in the organization of Army allergy. As has been previously stated, training camps present the allergist with the first real opportunity to observed adequately the allergic patient. The allergist at such camps should be well trained not only in the routine diagnostic and therapeutic measures pertaining to allergy, but he should also have available all pertinent information concerning possible allergic factors present in all theaters of operation and in all branches of the arms and services. If persons suffering from allergic diseases are adequately dealt with by such specialists, the problem of allergy in forward echelons would be of slight significance.

However, it is inevitable that the allergic diseases of some persons will not manifest themselves until after the soldiers have left the training centers. Such cases can best be handled at the large general hospitals where competent allergists should be stationed. There the duty of the allergist should include the routine care and disposition of allergic patients, and it also should be incumbent upon him to collect pertinent data relating to the various allergic

factors of those areas which his hospital services. These data then could be gathered by a board or committee of allergists in the office of the Surgeon General, and subsequently evaluated and distributed to the various hospitals having an allergy service. Thus, without the necessity of maintaining a large organization, it would be quite simple to collect and to disseminate significant information concerning allergic factors present throughout many areas. The function of such a board or committee would be not only to correlate scientific data relating to allergic diseases but also to standardize material and procedures to be used in the diagnosis and treatment of allergic cases. Testing material would conform to specific standards, thereby eliminating variations found in the different extracts now being utilized. Accompanying the standardization of diagnostic procedures would be the realization that military needs must be of primary consideration. In this way, adequate and reliable data can be compiled, evaluated, and used for the purpose of solving the problem of allergy in the Army. Chart 1 diagrammatically represents the organization described.

CHART 1



As military allergy gradually becomes more comprehensible, definite physical standards for admission into the Army can be promulgated. The use of such standards would eliminate the necessity of having highly trained allergists present at induction centers. With the passage of time the field of allergic

diseases assumes greater importance in medicine. It thus becomes incontrovertible that the problem of allergy is one that must be met and solved.

SUMMARY

From this article, which is an attempt to analyze the problem of allergy confronting the Army Medical Corps, certain conclusions may be drawn.

1. Over 1 per cent of all soldiers entering the armed forces require allergic care.
2. The problem of allergy in the Army may be divided into three categories:
   a. The professional duties of the Army allergist.
   b. The organization of an allergy section in the Army Medical Corps.
   c. The formulation of specific physical standards for all major allergic diseases.
3. The professional duties of the Army allergist consist of:
   a. Diagnosis and prognosis of the capabilities of the allergic soldier.
   b. Treatment of allergic cases giving sufficient consideration to the allergic problems which confront the ophthalmologic, otorhinolaryngologic, and dermatologic sections of the Medical Corps.
   c. Supervision of vaccine therapy.
4. The organization of an allergy department is suggested. This organization should consist of first, a board of allergists to control activities, to collect, correlate and disseminate pertinent data and to standardize all material and procedures to be used in the allergy department; second, allergy sections should be established at training centers and general hospitals, the former to prognosticate and classify allergic patients according to their capabilities, and the latter to collect significant data relating to the district they service and to handle cases arising after soldiers leave the training centers.
5. Inasmuch as the board at the office of the Surgeon General is the only body having available to it all the statistics regarding allergic diseases in the Army, it is that board which should formulate the specific physical standards to guide these Army physicians seeking to arrive at a solution of the problem of Army allergy.

REFERENCES

1. The Medical Department of the United States Army in the World War, Vol. 15, Statistics, Government Printing Office, Washington, 1925.
2. Vaughan, Warren T.: Problems of Allergy in War Time, Mil. Surgeon 89: 737, 1941.
3. Office of the Surgeon-General, Washington, D. C.: Reply to Communication dated October 16, 1942.
4. Blank, Philip: A Survey of Allergy in a Station Hospital, Mil. Surgeon. In press.

HEART DISEASE IN MIDDLE AGE*

LIEUT. COM. CHRISTOPHER C. SHAW, M.C., U. S. N. R.

IT HAS been said that "life begins at forty" but it might better be said that "life begins to go downhill at forty," for heart disease resulting from degenerative changes in the cardiovascular system is the most common, single cause of death and disability in the middle-age group between 40 and 65 years.[1] Disorders of the cardiovascular system which occur during middle life may be classified under three main headings:

1. Heart disease caused by infectious agents.
2. Heart failure resulting from metabolic and endocrine dyscrasias.
3. Cardiovascular disorders due to degenerative processes.

Within the first division one finds lesions of the cardiovascular system resulting from typhoid fever, syphilis, diphtheria, scarlet fever, bacterial endocarditis, glomerulonephritis, pyelonephritis, tuberculosis, and lead poisoning.

Of these infectious diseases involving the heart and blood vessels during middle age, syphilis is of prime importance. The Spirochaeta pallida attacks the myocardium and aorta and larger arteries in the general systemic invasion which follows shortly after the initial lesion, or chancre, appears. The organisms are distributed throughout the body and remain quiescent in the cardiovascular tissues during the long period of latency which precedes the onset of tertiary syphilis. When the signs and symptoms of cardiovascular lues appear they may develop with almost explosive violence.

The pathology in the myocardium consists of either a diffuse inflammatory involvement of cardiac muscle tissue with progressive congestive failure or the development of gummata with destruction of the intrinsic nervous pathways and subsequent arrhythmia, often in the form of heart block. The diffuse myocarditis alone may be a cause of sudden death in middle age.[2]

Invasion of the arterial tree by the spirochete of lues centers about the root of the aorta and begins as an obliterating arteritis of the vasa vasorum with subsequent necrosis and degeneration of the media of the aorta (a mesaortitis). This process continues and may cause formation of an aneurysm, or produce insufficiency of the aortic valve, or encroach upon the ostia of the coronary arteries which arise in the sinuses of Valsalva.

Aneurysmal dilation of the aorta may be either saccular or fusiform but in either case places practically no burden on the heart. Aneurysms cause symptoms only when they erode bone—ribs, vertebrae or sternum—or impinge upon vital structures in the mediastinum. Their presence, however, is a harbinger of death due to the danger of rupture with sudden exsanguination, or

*Reprinted from U. S. Nav. Med. Bull. 40: 579 (July), 1942.

the possibility of slow hemorrhage from necrosis and perforation of the aneurysmal wall. Insufficiency of the aortic valve and coronary stenosis produce typical clinical syndromes and require no further elaboration at this point.

Unfortunately for the patient and for the clinician, cardiovascular syphilis frequently develops in the presence of a negative Wassermann so that the diagnosis (and treatment) cannot be established by serologic tests. This probably occurs in 15 per cent to 20 per cent of luetic patients with cardiovascular syphilis, and is most likely the result of inadequate therapy during the primary and secondary stages of the disease. The treatment received was sufficient to reverse the positive Wassermann at that time but insufficient to prevent the development of cardiovascular involvement 10 to 20 years later, in spite of the fact that the patient's blood serology continued negative. In such instances, the doctor must lay great stress on the history of venereal infection and specific antiluetic therapy in youth and not be guided too strongly by repeatedly negative blood reports during middle age. Treatment should be directed toward alleviation of the congestive failure first, and then supplemented by careful administration of spirocheticidal drugs. The prognosis is poor in any form of cardiovascular syphilis.

With the exception of disease of the thyroid gland, metabolic and endocrine disturbances such as gout, obesity, pituitary, and adrenal tumors are relatively rare in older pilots and for this reason will not be considered in this discussion. Thyrotoxicosis, however, produces certain disturbances in the circulation which are worthy of comment.

The increase in metabolism due to an altered or toxic secretion of the thyroid hormone whips up the general circulation, causes slight peripheral dilatation, and increases the pulse pressure. The metabolism of the heart muscle itself is also elevated and results in a marked rise in the pulse rate with proportional decrease in the length of the diastolic phase of the cardiac cycle. The combination of these factors throws a tremendous load on the myocardium which will eventually show signs of congestive failure unless and until the toxic secretion is reduced by administration of iodine and then ablated by subtotal thyroidectomy.

In deficiency of the thyroid gland the patient becomes myxedematous, and as a result of the lowered metabolism the myocardium becomes soft and flabby and sluggish and unable to maintain an adequate circulation. Congestive failure will result but can be promptly mended by administration of thyroid extract by mouth. The basal metabolic rate is an index of diagnosis and of adequate thyroid medication in such cases. Sudden cardiac death has been reported in untreated myxedematous patients.[2]

Finally, mention must be made of the importance of masked hyperthyroidism in heart disease.[3] This possibility should be borne in mind in all cases of congestive failure which fail to respond to bed rest and adequate doses of digitalis. Such patients often exhibit transient auricular fibrillation, nervousness, loss of weight, tremors, and excessive perspiration and yet they present no exophthalmos nor enlargement of the thyroid gland. Blood pressure readings, pulse pressure, and pulse rate are frequently found to be within normal

limits in spite of the dyspnea, cyanosis, and edema which alarm the patient and confuse the physician. Low-grade glycosuria and mild diarrhea may also be present.

The basal metabolic rate in such cases will be elevated to +30 or even to +70 and will determine the true cause of the failure of the heart.[3] When masked hyperthyroidism is engrafted upon a heart already embarrased by previous disease—rheumatic fever with mitral stenosis or coronary occlusion with infarction—the differential diagnosis requires clinical acumen of a high order. The first syndrome may dovetail into and almost completely obscure the second. When the two conditions are found to coexist—based on repeated basal metabolic rate determinations—treatment should be directed toward the control and removal of the "masked" thyrotoxicosis.

By far the largest and most difficult field of heart disease in middle life comes under the heading of the "degenerative processes." This is especially true since the exact cause, or causes, of vascular degeneration are not known. However, because of the magnitude and importance of the problem, a short review of the salient points may not be amiss, even though our present knowledge of the subject is far from complete.

Degenerative diseases of the cardiovascular system include arteriosclerosis, coronary disease and angina pectoris, hypertension, and hypertensive heart disease. In this same category most authors include *cor pulmonale* and chronic nonvalvular heart disease, or chronic myocarditis.

Recent studies indicate that the underlying factors in all these degenerative states are heredity and the wear and tear of existence caused by the physical, mental and nervous strains of living and making a living in the twentieth century. The adage, "a man is as old as he feels," might be more truthfully paraphrased to read, "a man is as old as his arteries," that is, as old as the quality and function of his vascular tree inherited from his ancestors.

At the present writing, medicine has no control over heredity, although it is constantly striving to patch up inherited defects. By the same token, little can be done to control the speed of modern living or to lessen the wear and tear on the cardiovascular system incident to high-speed flying in modern military aviation. Both the strain and the stress are plunging forward at relatively increasing velocities and show no immediate evidence of reaching a reasonable equilibrium. And the cardiovascular system pays the piper in the long run. Sometimes the nervous system goes bankrupt first; but even in this case, the scars of battle have already left their indelible mark on the heart and blood vessels.

The most frequent "mark" is the atheromatous plaque of arteriosclerosis which is a manifestation of senescence of vascular tissue. The underlying cause probably will be found in a faulty metabolism of cholesterol, as it has been shown that the cholesterol content of the thoracic aorta increases with age.[4] This degenerative process termed "atherosclerosis" may be general in distribution involving all the arteries of the body from the largest to the very smallest, or it may be localized in one or more specific areas such as the brain, the coronary vessels of the heart, or in the kidneys.

As the vascular degeneration progresses, there develops a relative ischemia of the tissue supplied by the atherosclerotic vessel. If the process is gradual, a more or less efficient collateral circulation may be established which will serve to mask for a time the insidious and progressive nature of the atheromatosis. On the other hand if the diminution of blood flow to a part or organ be sudden or complete, as in thrombus formation over an ulcerated sclerotic plaque or in fracture of a calcified atheroma with embolic phenomenon, then infarction of the tissue peripheral to the sudden occlusion results. In either instance the end picture is primarily a diffuse or localized fibrosis of the parenchyma.

Vascular degeneration in the brain caused by atherosclerosis gives rise to the signs and symptoms of cerebral arteriosclerosis. In the kidney the disease may remain quiescent until the rather sudden onset of marked renal impairment or progressive renal insufficiency appears.[2] Arteriosclerosis of the vessels supplying the pancreas gives rise to a true diabetes which is usually comparatively mild. In the extremities the process produces causalgia and intermittent claudication. Thrombosis and/or embolism rapidly lead to gangrene.

Arteriosclerotic aortitis is usually symptomless until the process invades the ring of the aortic valve causing stenosis, or unless the coronary ostia in the sinuses of Valsalva are impinged upon by atherosclerotic plaques. An aneurysm of the aorta due to arteriosclerosis may be a simple, diffuse, silent dilatation or it may occur as the dissecting type which is spectacular, excruciatingly painful, and usually causes sudden death from shock or from rupture of the aorta in a few hours or days after the sudden and dramatic onset of the dissection of the media.

Atheromatosis of the coronary vessels leads to coronary insufficiency and occlusion with impaired cardiac efficiency due to myocardial ischemia and infarction. In this connection it is interesting to point out that coronary occlusion *per se* does not necessarily produce any characteristic clinical manifestation.[5]

If the occlusion develops very gradually (as it may in atherosclerosis), nature will improve the opportunity to develop or expand the potential collateral circulation in the heart muscle and produce anastomoses sufficient to prevent, or circumvent, infarction of the myocardium. This is brought about by opening of the Thebesian capillaries coursing within the muscle bundles and by the propagation of vessels from the circulation of the pericardium at its attachment near the base of the heart.

The extent and efficiency of this collateral circulation have not been generally realized, but its importance is evidenced by the fact that it is found operative in approximately one-fourth of the patients with coronary occlusion.[1] Their lives have been prolonged for years and they may have remained symptomless because of the very chronicity of their vascular degeneration. At postmortem examination extensive atherosclerosis of the coronary vessels may be present out of all proportion to the clinical manifestations.[6] Cases are on record in which one or both coronary arteries have been found completely occluded at autopsy, yet the patient remained free of symptoms referable to the heart during life and succumbed from noncardiac causes.[1]

If, on the other hand, the occlusion occurs rapidly, the classical picture of myocardial infarction develops. This is characterized by substernal pain and oppression due to anoxia of the infarcted area of heart muscle.[7] There is concomitant fall in blood pressure, pallor, shock, fever, leucocytosis, pericardial friction rub and typical electrocardiographic changes. In such cases the clinical diagnosis is usually coronary thrombosis, but the term "myocardial infarction" would be more accurate.

Pain is commonly an outstanding symptom of this syndrome and frequently is crushing or grinding in severity and of prolonged duration. In other cases pain may be notable by its absence, since there are certain areas along the lateral wall of the left ventricle which are relatively silent as far as symptoms and signs and electrocardiographic evidence of infarction are concerned. Many cases of painless myocardial infarction have been recorded in which the only complaints were sudden dyspnea and progressive congestive failure.[8]

Sclerosis of the peripheral vessels is not a true cause of heart failure, for it has been shown that extreme degrees of peripheral arteriosclerosis have no deleterious effect on cardiac function;[3] nor is there necessarily any relationship between peripheral and coronary vascular degeneration. The former may exist in the entire absence of the latter, and, conversely, coronary atheromatosis may be present to the point of death and show no evidence of narrowing or tortuosity or induration of the peripheral arteries, nor of the retinal vessels in the fundi. The term "arteriosclerotic heart disease" is, in a sense, a misnomer and should be dropped in favor of the more truly descriptive phrase "coronary artery heart disease."[3]

Angina pectoris manifested by substernal or precordial pain of agonizing character and short duration is a symptom complex and not a disease entity. It is the consensus that it results from anoxia of the heart muscle[7]—in other words, a cardiac "cramp"—which may be brought about by angiospasm or by partial occlusion of a coronary artery or one of its branches. Vasospasm occurs in about 10 per cent of patients suffering with angina and is induced by hypersensitive nervous mechanisms, by thyrotoxicosis, by ingestion of certain poisons, and by excessive inhalation of nicotine. Coronary thrombosis with anginoid pain is three times more prevalent in heavy smokers than in nonsmokers during middle age.[1] In 90 per cent of the cases diagnosed "angina pectoris," degenerative disease of the coronary arteries can be demonstrated.

A word of caution in regard to the close association between arteriosclerosis and hypertension. It is definitely accepted that arteriosclerosis does not produce hypertension. Hardening of the peripheral vessels alone is conducive to hypotension rather than high blood pressure since the elastic recoil of the arterial wall is greatly reduced by atheromatous infiltration of the intima. Arteriosclerosis may, therefore, exist for years without any rise of blood pressure. On the other hand, hypertension cannot be present for long without producing degenerative changes in the vascular tree. Hence it is difficult to say whether the high blood pressure caused the arteriosclerosis or whether the

unknown cause, or causes, of the hyperpiesis exerted a concomitant deleterious effect on or within the intima of the blood vessels.

Be that as it may, the actual cause of hypertension still remains a mystery. Numerous theories have been applied to the problem but all have been found wanting. The most rational hypothesis deals with the irritability of the arterioles causing vasospastic constriction which produces a proportional increase in peripheral resistance and this in turn necessitates an increase in hydrostatic force—that is, an elevation of the blood pressure—to cope with the augmented or heightened resistance in the peripheral circulation.

During the past decade considerable experimental work has been done on this problem, and it has been shown repeatedly that experimental hypertension can be produced by renal ischemia.[9-12] Furthermore, this rise in blood pressure (produced experimentally) is of humoral origin and arises as a result of the presence of an excessive amount of pressor substance in the circulation. This pressor substance is not actually produced in the ischemic kidney but rather is a combination of a precursor (renin) liberated by the damaged kidney and an activator (angiotonin) already in the blood.[13] Moreover, this pressor substance (hypertensin) acts directly on the arterioles to produce vasoconstriction. The clinical application of this recently acquired knowledge is, like prosperity, probably "just around the corner." In the meantime the clinical problem of hypertension—both the benign and the malignant type—continues to baffle the internist.

A confusion of tongues has arisen in the matter of nomenclature. The terms "malignant sclerosis," "malignant hypertension," and the "malignant phase of essential hypertension" all refer to the same syndrome. High blood pressure is not a disease entity but rather a symptom complex in which the hypertension is either primary (essential) or secondary to any one or any combination of a number of different diseases. This concept is not generally entertained but its validity is attested clinically by the fact that in the long run the *modus operandi* of every case of hyperpiesis is identical, no matter what its pathogenesis is or may have been. The malignant phase frequently develops after hypertension in the benign form has been present for a variable length of time, perhaps even unsuspected.

In a small percentage of patients hyperpiesis may apparently be malignant from its onset. This is termed the *de novo* form of the disease and may occur explosively in certain cases of primary hypertension or develop quite dramatically in patients who for many years have suffered from latent renal disease without any previous increase in their blood pressure.[14]

The onset of the malignant phase of hypertension (be it "primary" or "secondary") is characterized by severe headache, visual disturbances, vomiting, anorexia, cachexia, and exhaustion. These signs and symptoms in the presence of a systolic pressure bordering on 200 mm. Hg and a diastolic pressure in the neighborhood of 110 mm. Hg, or higher, predict a progressively rapid failure of the cardiac or renal functions. Patients with hypertension are all potential cardiacs or nephritics. Their clinical course is downhill (rapid in the essential or malignant type; slow in the benign or secondary form) and almost always terminates fatally in cardiac failure, or in apoplexy, or in uremia.

Patients suffering and dying from hypertension, or the effects thereof, can be segregated into several well-defined etiological and pathological groups, depending on the pathogenesis of their underlying disease, as follows:[14]

1. Primary or essential hypertension (cause, or causes, still in doubt).
2. Secondary hypertension due to—
   A. Renal disease:
      a. Glomerulonephritis.
      b. Pyelonephritis.
      c. Intercapillary glomerulosclerosis.
      d. Congenital unilateral hypoplasia.
      e. Obstruction of renal artery by atherosclerotic plaque.
      f. Multiple occlusions of small renal arteries.
      g. Renal infarct.
      h. Periarteritis nodosa of renal vessels.
      i. Polycystic kidneys.
   B. Chronic lead poisoning.
   C. Polycythemia vera; leucemia.
   D. Endocrine disease.
      a. Pituitary—basophilic adenoma.
      b. Adrenal—hypernephroma; paraganglionoma.
      c. Thyroid—thyrotoxicosis.
      d. Hyperemesis gravidarum.

Time and space do not permit further elaboration of the factors listed in the table. During life it is usually impossible to tell whether the patient is suffering from hypertension which is primary unto itself or is secondary to some unrecognized morbid lesion. Whatever the nature of the underlying pathological process, the prognosis is poor either in the long or the short run. Treatment is purely palliative except in those rare cases where the renal or glandular pathology is amenable to surgery. The results of sympathetic surgery in patients with the malignant form of hypertension have been uniformly disappointing.[15]

The term *cor pulmonale* refers to failure of the right ventricle as a result of increased pressure in the pulmonary circuit. This may occur in the acute form following a large pulmonary embolus but more frequently is a chronic affair as seen in emphysema, silicosis, stenosis of the pulmonary artery, and in extensive fibrotic tuberculosis, or in spontaneous pneumothorax (especially when bilateral). These conditions, when they do develop, usually appear during middle life.[2]

Cardiac disease caused by avitaminosis and dietary deficiencies, such as protein starvation, is rare in this country but is a possibility among the civil population during wartime. Myocardial failure due to the presence of a peripheral arteriovenous fistula—the traumatic variety should present no diagnostic difficulty—to tumors of the heart muscle and to deformities of the spine and thoracic cage is so rare that it will practically never enter the physical picture of the aviator approaching middle age.

There is one form of chronic myocardial insufficiency, however which deserves mention because its etiology is not clear, because it is frequently overlooked, and because its onset is insidious and its course progressive.

This is a nonvalvular cardiopathy characterized by congestive failure of unknown cause. Clinically, there is no evidence of hyperthyroidism or emphysema or avitaminosis or protein deficiency or blood dyscrasia. Neither are there any signs or symptoms of infection of the endocardium, myocardium, or pericardium. Nor can vascular degeneration nor hypertension be indicted—and yet the myocardium tires out during middle age, either early or late, and runs down like an alarm clock and stops.

At autopsy, the heart is found to be both dilated and hypertrophied, but the heart muscle otherwise appears normal to all intents and purposes. For lack of a better name such cases have been termed "chronic myocarditis," which is another misnomer since no inflammation of the myocardium can be found. The term "chronic myocardosis" has been suggested[16] to account for the dyspnea on exertion, the flatulence after meals, and the mild precordial pain (transient but recurrent) which warn of impending congestive failure. These symptoms, however, are quite frequently found in many cardiac syndromes and should not be assigned (or consigned) solely to chronic myocardosis. It may be more satisfactory to designate this symptom complex by the term "nonvalvular heart disease," or simply "chronic myocardial insufficiency."

Since heart disease is so prevalent and ubiquitous in middle life and since it is the most frequent cause of death and disability between the ages of 40 and 65 years, its prevention and early detection are of paramount importance. This is especially true for the profession of aviation, for while it is significant that anoxia causes a rise in blood pressure and pulse rate equivalent to that produced by mild exercise only, it has been realized more recently that the chronic stresses of flying eventually induce a generalized (and rapid) deterioration of the cardiovascular system.[17]

In most instances this premise applies, not only for the aviator but for the entire personnel of the Navy. In the past 20 years, the Bureau of Medicine and Surgery has recorded an alarming increase in coronary disease. The age incidence in these coronary deaths is lowering year by year and the morbidity is getting higher.[18] McIntire[18] states:

> Are we crowding our officers to a point where the cardiovascular system can no longer carry its load, or are we piling on so much responsibility that the nervous system cannot maintain its stability? . . . In the Navy a man must be physically fit to perform his duties at all times, in all places and under any condition. . . . It is the duty of the internist to find means of preventing disabilities, especially those due to degenerative disease.

While the call to duty in this respect is clear, the means of fulfilling this phase of our mission still remain obscure. This, in part, may be due to the fact that many of the methods and instrumentalities of modern cardiology lack precision. Some of the problems in this field of internal medicine yield only to empirical solution—or remain unsolved. Others are forced to rely for an answer almost entirely on the patient's history, which may be accurate and intelligent, yet incomplete; or it may be inaccurate and, therefore, worthless. Many of the signs and symptoms are transient; others notoriously absent when needed most to clarify the diagnosis. Even our present laboratory methods are far from infallible. Much must still be taken (and given) "on faith."

Pending that happy day when detection, recognition, prevention, treatment and after-care of the patient with degenerative cardiovascular disease shall reach a more rational (and simpler) plane, we must use our present scanty knowledge of the problem to the best possible advantage. Many time-honored procedures have proved their worth and have behind them the authority of regulations; other and newer concepts and diagnostic methods deserve consideration and fair trial.

Determination of the fitness of the cardiovascular system for continued military service is difficult because the examiner must rely chiefly on signs alone, and often these signs are of questionable significance. Tachycardia, systolic murmurs, slight elevation of blood pressure may attract the attention of the examining physician. Usually these findings are of no particular importance, yet their presence demands careful investigation. Is the tachycardia due to nervousness or to the presence of a "hidden" infection (notably tuberculosis)? Is the elevation of blood pressure relative or absolute? Is it a precursor of essential hypertension? Does poor response to exercise indicate an impending effort syndrome? How can these questions—and a host of others—be answered unequivocally?

In an attempt to arrive at a reliable evaluation of the cardiovascular system of the middle-aged flier one must first have a base line on which to "base" his judgment. This may date back to the general physical examination at the time of his enrollment or commission.[19] In addition to the routine Schneider test and examination of the ocular fundi, an electrocardiogram and teleroentgenogram of the heart would be desirable. The cold pressor test and "circulation time" may provide important information on the "tone" of the peripheral vascular tree. Annual fluoroscopic examination of the heart and aorta and routine electrocardiogram every year after the age of 40 may some day be included in the *Manual of the Medical Department.*

In regard to the strain of modern living and the extra stresses placed upon the heart and blood vessels by commercial and especially by military aviation, little can be offered other than education and advice concerning the value of rest and the art of relaxation while off duty. Excessive indulgence in alcohol, tobacco, and other forms of dissipation are no respecters of persons or occupations. Such habits probably hasten the onset and speed the progress of degenerative diseases of both the cardiovascular and nervous systems.

The personnel of our armed forces who are approaching middle age must learn to acknowledge and respect their physical and nervous limitations. To grow old gracefully and graciously still remains an art which is worthy of cultivation and patronage. By so doing, our officers and men will be enabled to render better service to our country and live longer, more useful, and happier lives.

BIBLIOGRAPHY

1. White, P. D.: Heart Disease. New York. 1937, Macmillan Co.
2. War Department: Notes on Cardiology in Aviation Medicine, Technical Manual 8: 305, 1940.
3. Levine, S. A.: Clinical Heart Disease, ed. 2, Philadelphia & London, 1940, W. B. Saunders Co.
4. Bruger, Maurice, and Chassin, M. R.: Cholesterol Content of the Renal Arteries and of the Thoracic Aorta in Relation to Hypertension and Vascular Pathology, Ann. Int. Med. 14: 1756-1767, 1941.

5. Stroud, W. D., and Wagner, J. A.: Silent or Atypical Coronary Occlusion, Ann. Int. Med. 15: 25-32, July, 1941.
6. Smith, F. M.: Coronary Artery Disease: the Diagnosis and Treatment of Cardiovascular Disease, Philadelphia, 1940, F. A. Davis Co., p. 387.
7. Levy, R. L.: The Diagnosis of Coronary Insufficiency; the Diagnosis and Treatment of Cardiovascular Disease, Philadelphia, 1940, F. A. Davis Co.
8. Boyd, L. J., and Werblow, S.: Coronary Thrombosis Without Pain, Am. J. M. Sc. 194: 814-824, December, 1937.
9. Goldblatt, H.: Experimental Hypertension Induced by Renal Ischemia; Harvey Lectures, Baltimore, 1937-1938, Williams & Wilkins Co.
10. Smith, H. W., Goldring W., Chasis, H., and Ranges, H. A.: Observations on the Effective Renal Blood Flow and Functional Excretory Mass in Man, With Special Reference to Essential Hypertension, Am. Jour. Physiol. 123: 189, 1938.
11. Grollman, A., Williams, J. R., Jr., and Harrison, T. R.: Reduction of Elevated Blood Pressure by Administration of Renal Extracts, J. A. M. A. 115: 1169-1176, October 5, 1940.
12. Page, I. H.: Newer Aspects of Experimental Hypertension; Blood, Heart, and Circulation, Am. Assn. Adv. Sc. Pub. No. 13: 239, 1940.
13. Smith, D. C.: Experimental Renal Hypertension, Bull. School of Med., University of Maryland 26: 1-10, July, 1941.
14. Derow, H. A., and Altschule, M. D.: The Nature of Malignant Hypertension, Ann. Int. Med. 14: 1768-1780, January, 1941.
15. Page, I. H.: Medical Aspects of Surgical Treatment of Hypertension, J. A. M. A. 110: 1161-1165, April 9, 1938.
16. Hyman and Parsonnet: The Failing Heart of Middle Age, Philadelphia, 1935, W. B. Saunders Co.
17. Armstrong, H. G.: Principles and Practice of Aviation Medicine, Baltimore, 1939, Williams & Wilkins Co., p. 110.
18. McIntire, R. T.: The Problem of the Internist in the Navy, Ann. Int. Med. 14: 2179-2183, June, 1941.
19. Naval School of Aviation Medicine, Pensacola, Florida: Physical Examination Manual, 1941.

RESPONSIBILITIES OF THE ARMY ROENTGENOLOGIST*

Lieut. Col. Alfred A. de Lorimier, M.C., U. S. Army

NOT infrequently the statement is made: "Roentgenology is roentgenology whether it be practiced in the Army or out of the Army." At first thought, this expression might seem to specify adequately the situation. In many respects it is true.

Scientific practice of roentgenology requires deliberative analyses of projected images on films or a fluoroscopic screen; a mental or recorded listing of tangible findings—the evidence described as "roentgen criteria"; an evaluation of such findings with respect to one or another pathologic entity; and finally, a correlation of the possibilities with consideration of the clinical aspects and laboratory studies. The roentgenologist must be recognized as a consultant. As such he must have a grasp of the entire field of medicine. He must exercise judgment just as required of a surgical consultant or any other type of medical adviser. These requirements are common both to the Army roentgenologist and to his confrere practicing this specialty in civil life.

There are a number of subspecialized qualifications frequently required of the roentgenologist practicing in civil life, which cannot be expected as demands upon the Army roentgenologist. For instance, rarely would the Army roentgenologist be concerned with pelvimetry, gynecologic roentgenology, or studies

*From the Department of Roentgenology, Army Medical School.

of infants and children. Moreover, in the theater of operations it can be expected that he will seldom have to deal with the treatment of malignancies—though this latter responsibility must be handled in certain general hospitals located in the Zone of the Interior.

These exemptions are more than offset by other requirements. The Army roentgenologist must possess an exceptional degree of ingenuity and the ability to command men and to coordinate very diplomatically the roentgenologic service; the ability to discern diagnostic criteria by way of roentgenoscopy without requiring film evidence; familiarity with foreign-body localization; special diagnostic acumen in the field of maxillofacial abnormalities; and a familiarity with requisitioning and handling of supplies.

Let us consider the magnitude of special qualifications such as these.

At a general hospital the roentgenologic service can be expected to be similar to that of any large civilian hospital. Similar services might be required at a station hospital.[1] However, at a fixed or a motorized evacuation hospital, at a surgical hospital, at a convalescent hospital, or on a hospital transport ship, in any of these locations, it can be expected that the roentgenologist will have to assemble his equipment; he will have to adjust it to one and another type of electrical supply, possibly disassemble it, move to a new location, and repeat this process over and over. In most instances, when trouble develops he will have to analyze the nature of the trouble and probably repair it himself. True enough, he will have assistance by way of enlisted technicians, but in most instances such assistants will be of unknown trust, and even though eventually trained they must be considered as transient. At best, he will be dealing with strangers much of the time. True enough, there will be repair centers, but these may be located at a considerable distance and available only after much delay. Therefore, the Army roentgenologist must develop himself to a standard of self-reliance, not only with respect to diagnostic problems but also in the handling of equipment.

In this connection it might be mentioned that the type of electrical currents available in one or another part of the world is very variable. The conventional current as found in the United States, 110 volts, 60 cycle, single phase, is not available widely throughout the rest of the world. In some locations, even here in the United States, 50 cycle current is used; in a few locations, it is a 30 cycle current. In our own Canal Zone it is 25 cycle. In most parts of the world, 50 cycle current, with a voltage of 200 to 260, is supplied the communities. Moreover, the most common type is three phase rather than single phase.

It has not appeared to be practical to provide means for altering the cycle such as might be available in one or another locality, since equipment required for such conversions would have to be very bulky and very heavy. However, it has been deemed practical to provide for adjustment of the voltage and the phase. This can be accomplished by use of a portable autotransformer. This item weighs approximately 80 pounds. It has a 70 foot line cable of a gauge satisfactory to handle relatively high wattage under conditions of voltages of the 200 to 260 range, and its design is such that it can be connected into the higher voltage line and deliver the lower voltage for which the portable field x-ray equipment and many other electrical devices have been designed. It may

be connected into two leads of a three-phase circuit so as to deliver single-phase current. Fortunately, the Army field equipment has been designed for operation on either 50 or 60 cycle current. Thus, with the aid of this autotransformer, our field x-ray equipment can function in most parts of the world. Where the cycle is less than 50, a special portable gasoline-electrical generator, having a capacity for 2,500 watts, may be used. Larger capacity mobile generators are

A.

B.

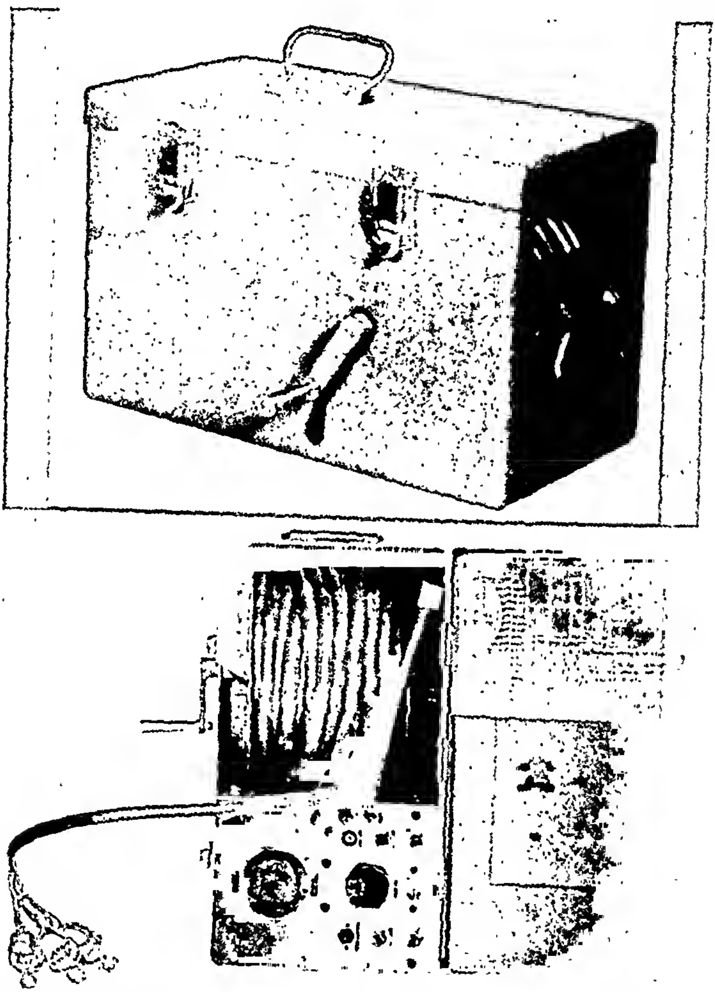


Fig. 1.—The portable autotransformer unit. *A*, Showing packing arrangement; *B*, top opened, showing 70 foot cable ready for fixation to open leads such as available in a distribution box. Note the meter for indicating the voltage applied to electrical apparatus connected into the secondary circuit of the autotransformer winding.

provided wherever needed. The requirements can be expected to differ in one and another location, and constantly it will be necessary for the roentgenologist to deliberate as to what adjustments are indicated. In these problems he cannot depend upon factory personnel or local electricians.

All field x-ray equipment has been designed on the basis of several limiting axiomatic principles.[2] All of it is portable. That which is to be used farther forward than "fixed types" of evacuation hospitals is packed in field chests.

Originally, it was intended that no one of these chests when packed should weigh more than 200 pounds. Because of various requirements including limitations on priority of materials, it has been necessary to deviate from this fixed policy. However, no one chest packed weighs more than 357 pounds (the gasoline-electrical generator). All the chests are balanced and so constructed that they can be carried by two to four men. Each item has been designed for multiplicity of adaptation. Single purpose application has been considered impractical. Besides providing that each item be usable for many purposes, the policy has been to provide usefulness for any type of hospital installation and to meet any type of situation.

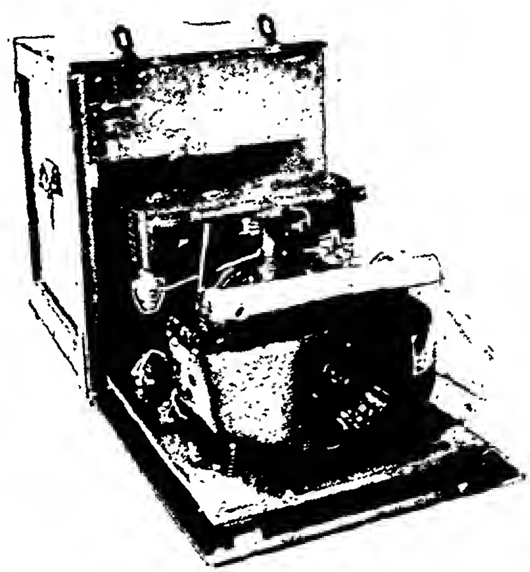


Fig. 2.—The portable gasoline electrical generator. Two gallons of gasoline provides for a supply of 2,500 watts continuously for approximately four hours.

For instance, the x-ray tent may be used for roentgenoscopy, with one arrangement of its inner curtain, or it may be used for film processing by merely changing the arrangement of its inner curtain and auxiliary drapes. Moreover, this tent may be erected by itself on the outside; it may be erected within another tent such as a ward or corridor tent, or it may be erected in any room or dugout. Under very windy conditions, it may be reinforced with the use of guy ropes. However, guy ropes and ground poles are not necessary. It is anticipated that in many instances dugouts or cellars will be utilized. The tent as well as all the other equipment can be adjusted quickly to such a situation. In fact, with forceful supervision and with very little training of three or four technicians, it is possible to move into a new location and to set up the entire equipment as needed for roentgenoscopy and to be ready to function within fifteen minutes after the time of arrival. This same equipment might be mounted onto closed trucks, thereby being made available for usage at a moment's notice.

The x-ray table unit, together with the x-ray machine unit and its chassis, has been designed for a nine-way adaptation. They may be adapted for horizontal roentgenoscopy; for a rapid roentgenoscopic method of foreign-body localization; for sitting roentgenoscopy; standing roentgenoscopy; horizontal roentgenography, with the tube above the table; horizontal roentgenography, with focal film distances as great as 6 feet, the patient being positioned on a litter at the floor

A.

B.

Fig. 3.—The darkroom tent: *A*, As erected for roentgenoscopy—note the forward position of the inner curtain. The litter bearers simply butt through four drapes. *B*, As erected for film processing—the inner curtain has been set back 22 inches; the inner drapes have been closed by way of a zipper so that the technician enters through labyrinth.

level; vertical roentgenography, using similar long focal film distances; bedside roentgenography (utilizing a mobile arrangement of the x-ray machine unit); and finally, this equipment may be used for roentgenotherapy for conditions requiring a quality of x-radiation, such as produced with a kilovoltage of 100—infections (including gas gangrene) and dermatoses.

All these applications must be understood, and the roentgenologist must be capable of quickly adjusting not only to new conditions but also to any of a

variety of demands with the minimum of equipment. In connection with foreign-body localizations, it should be emphasized that another item has been developed, a "biplane marker and reorientating device." This item is of a design such as to provide for its being adapted to the roentgenoscopic screen. It is designed to serve for spotting onto the skin of the patient, the location of the foreign body with respect to a plane at right angles to the plane concerned with the roentgenoscopic localization. This *second* spotting and localization are accomplished without any x-radiation exposure of the patient, and without the use of x-radiation at all. It provides for spotting of the position of the foreign body just as might be accomplished with the use of biplane roentgenoscopy. Yet, the shortcomings of biplane roentgenoscopy: the bulk of a second tube and second roentgenoscopic screen or the time delay concerned with shifting a single tube and screen from the first plane to one at right angles to it; difficulties of visualization of a small foreign body through great thicknesses and densities concerned with lateral planes, and the increased x-radiation exposures with respect to both the patient and the roentgenoscopist such as required for viewing in the two planes—all of these shortcomings are overcome with use of the biplane marker.

This "spotting" in the two planes serves for more definite guidance to the surgeon. It can be anticipated that the patient may be lying in one position during roentgenoscopy, whereas he might be rotated to a different position for the surgical approach. The surgeon may not appreciate these variations of relationship and he may, therefore, approach the foreign body along a plane at variance with the plane concerned with the alignment of the foreign body to the spotting on the skin surface. Perhaps, due to anatomic limitations, the approach may have to be from an altogether different plane relationship than either of two planes so far considered. In such cases, biplane marking itself should indicate the true relations and guide the approach.

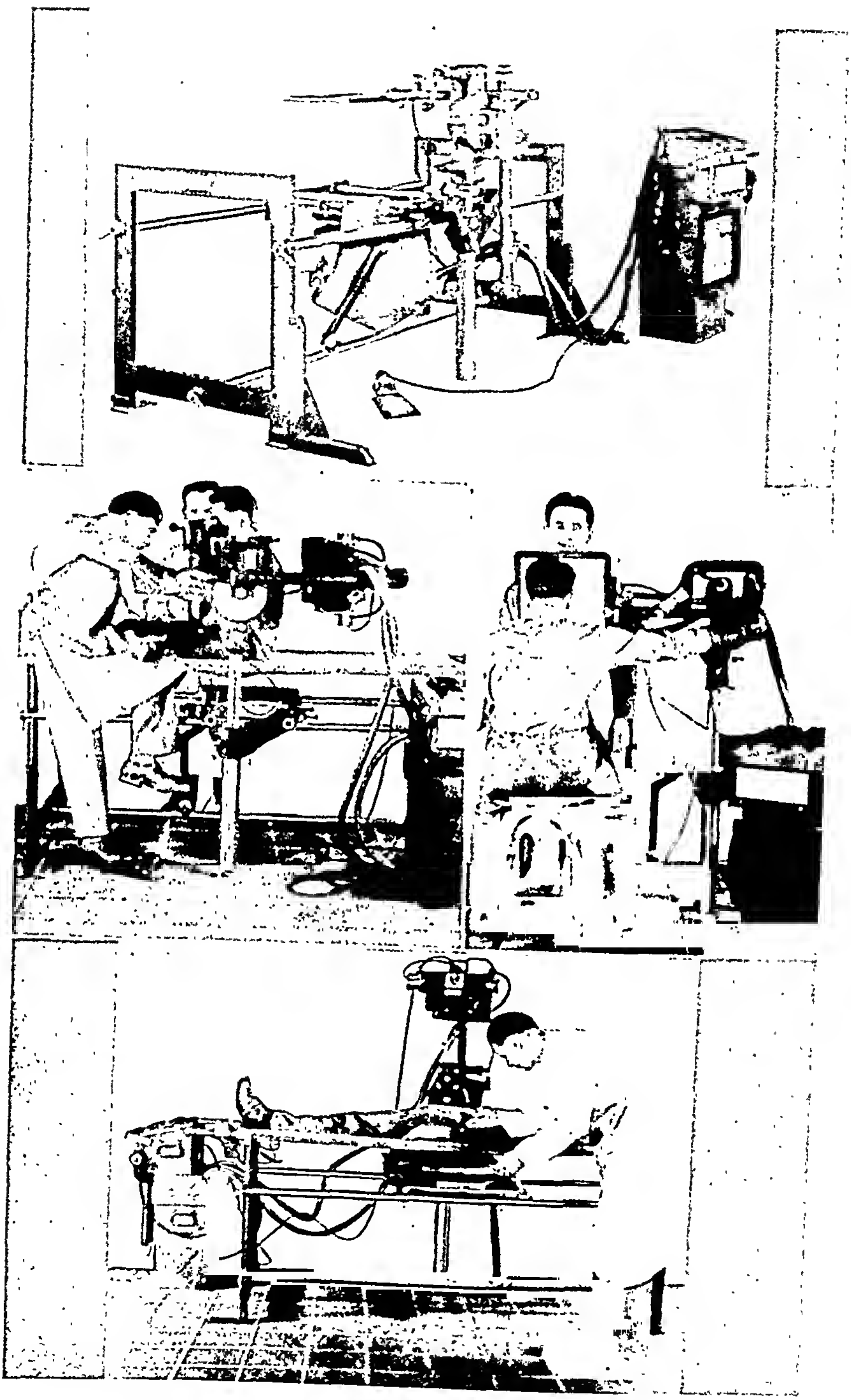
In some instances, it can be expected that the surgeon must be assisted even further. It may be necessary to re-establish more thoroughly the relations on the operating table, as they pertained during the localization procedure. For this, the two spottings on the skin surface already mentioned may be used in connection with the re-orientating device to indicate very definitely relations for proper positioning. This device contains three adjustable probe directors. They can be detached for sterilization purposes and if needed they can be repositioned onto their respective supports and used right in the operative field under conditions of surgical asepsis. One probe may be positioned for indicating the plane with respect to the spotting as accomplished with the roentgenoscopic procedure; a second one may be positioned for indicating the plane concerned with the spotting as accomplished with the biplane marker; the third probe may be positioned for directing the plane of any operative approach that the surgeon might have selected. Thus localization may be accomplished with respect to *any* plane. The roentgenologist is charged not only with the "spotting" of the foreign bodies and calculating depth values with respect to them, but he must actually coordinate with the surgeon in accomplishing their removal.

These procedures do not require the use of films. All of them can be accomplished within time limits of a few minutes. Actual energization of the x-ray equipment and radiation exposure should not exceed fifteen to forty-five seconds.

A.

B.

C.



D.

Fig. 4.—Adaptations of the x-ray machine unit. *A*, The set-up with the field table with arrangement of the tube and fluoroscopic screen for horizontal roentgenoscopy and for foreign-body localization; *B*, the arrangement for sitting fluoroscopy; *C*, the arrangement for standing fluoroscopy; *D*, over the table roentgenography; *E*, horizontal roentgenography with long focal film distance; *F*, vertical roentgenography with long focal film distance; *G*, adaptation of the x-ray machine unit to the chassis unit for mobile roentgenography or roentgenotherapy.

E.

F.

G.

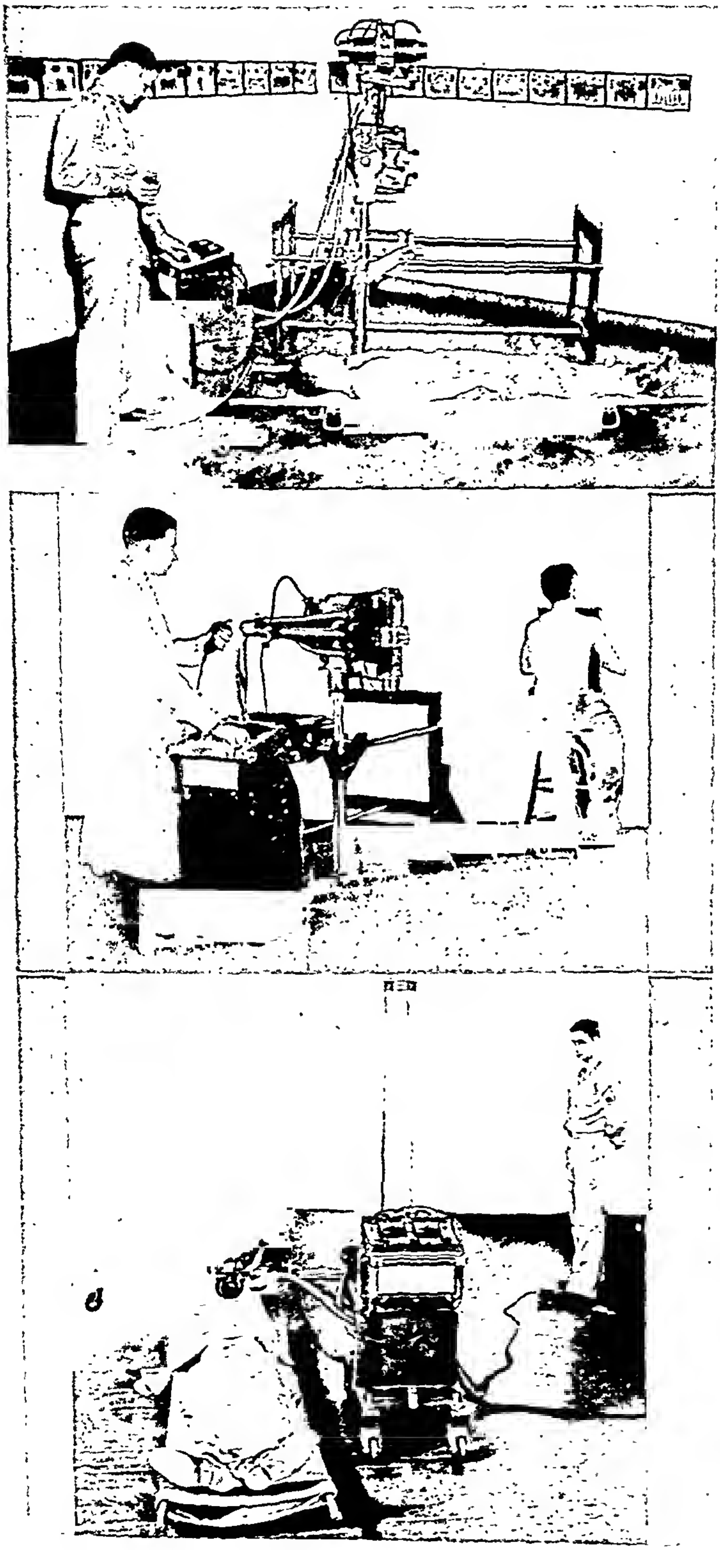


Fig. 4.

depending upon the experience of the roentgenoscopist. For precision, though, it is necessary that one be alert to conditions which might result in errors and that he be understanding of the means of correcting such. With each disassemblage and with each assemblage of the equipment following packing and transportation, at least minor damage, such as stripping of threads, perhaps bowing of the localization scale or bending of the depth marker, warping of the roentgenoscopic screen, etc., can be expected to occur. Unless the roentgenologist is constantly alert, such defects will lead to not one or a few mistakes but to a continuous repetition of mistakes.

A small depth phantom has been provided, this being supplied for the purpose of preliminary checking of the accuracy of the localization. Moreover, an adjustment means is incorporated into the supporting arm of the roentgenoscopic screen to correct for variations in focal-spot-roentgenoscopic screen distance due to abnormal positions of the focal spot itself. Adjustment of the reading level concerned with the depth scale has also been provided. This latter provides for cancellation of errors which would otherwise occur due to warpage or corrugation of the roentgenoscopic screen or to tangential viewing by one or another roentgenoscopist with resultant parallax. In short, provisions have been made to compensate for those variables which are beyond the scope of control by the manufacturers. These provisions theoretically mean absolute accuracy. Nevertheless, the degree of accuracy is dependent upon knowledge of the fundamentals, together with the integrity and scruple of the roentgenoscopist.

This general procedure of foreign body localization has been considered adequate to handle foreign bodies in all locations other than in the eye. For localization of intraocular foreign bodies, the "Sweet method," such as used in World War I, still appears to be the most practical. This method requires the use of film. Therefore, such localizations cannot be expected in the very forward zone. They will be accomplished in the more fixed types of evacuation hospitals and general hospitals, where films and processing equipment are available. This particular method is well known to roentgenologists in civil life. Detailed description of it, as well as explanation of the roentgenoscopic procedure, is published in the *Technical Manual 8-275*[3] and elsewhere.[4]

In the very forward installations, such as the surgical hospital, the roentgenologist is the sole diagnostician. In the motorized evacuation hospital, he is one of four diagnosticians. Internists and collaborative consultants are included with installations located farther to the rear. However, due to the requirement of having to arrive at quick decisions and, of having to handle so many hundred cases each day, the roentgenologist may be rated as one of the most useful consultants. He must be capable scientifically, and he must also be very ethical in dealing with his confreres. He must be a coordinator. The attitude of *service* must dominate his actions.

His coordinating spirit must not be limited to his own relations with respect to his doctor confreres. He must be able to command men. In certain locations, he may have only three technical assistants; in others, as many as ten or twelve. Under conditions of warfare, the doctor, the officer, and the enlisted personnel must work shoulder to shoulder. Nevertheless, the enlisted personnel must not be leaned upon too strongly. The roentgenologist must conservatively control the group.

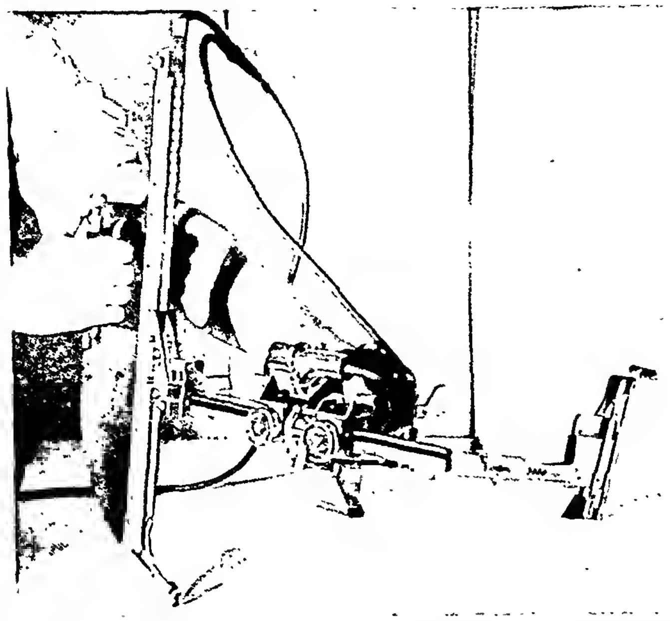


Fig. 5.—The biplane marker. The operator is about to fix this device to the roentgenoscopic screen. Marking in the second plane is accomplished without any x-radiation exposure. Note the pilot lights and cables, provided for manipulating this apparatus without losing accommodation of the darkroom.

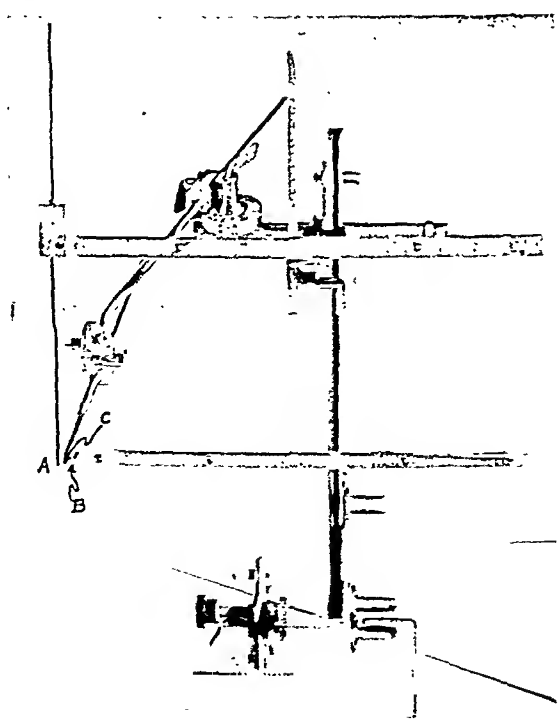


Fig. 6.—The reorientating device. Note the three pointers: *A*, Concerned with the spotting as accomplished with the roentgenoscopic procedure; *B*, that concerned with the spotting as accomplished with the biplane marker; and *C*, the indicator concerned with any selected surgical approach.

As mentioned in the beginning, the diagnostic responsibilities are almost of as great a scope as those with which the civilian roentgenologist has to deal. There will be need for roentgenoscopy of the extremities, with interest as to soft tissue wounds—possibly with interest as to gas bacillus infections. There will be problems relative to fractures; these under conditions of fixation with splints or plaster casts. There will be problems pertaining to wounds in the abdomen and thorax, and there will be problems concerned with a gamut of pathologic conditions besides those of trauma for any of these parts. True enough, similar diagnostic problems are experienced by a well-practiced roentgenologist in civil life. However, in Army roentgenology it can be expected that roentgenoscopy will have to be emphasized over roentgenography. Resort to films requires more time than can be expected to be allowable during stress of activities such as are to be expected in the theater of operations. The roentgenologist must have developed a very keenly discerning eye. Of all phases of roentgenology, it would seem that the field of maxillofacial abnormalities must prompt his particular study and proficiency. Tank and air service casualties, as well as those concerned with ordinary motor transportation, must be expected. There are so many contours concerned with the facial bones and the cranium itself that distinct visualization of any one portion of the head is a difficult task under any conditions. The Army roentgenologist must be thoroughly understanding of all of these contours and of the several positionings concerned with most adequately visualizing one or another portion. He must have a knowledge of anatomic weak sites. He must be equipped with a knowledge of the physiology concerned with the various muscle actions which might serve to alter relations in case of fracture in one or another location. He must be able to discern roentgenoscopically these conditions which are admittedly difficult of diagnosis even with the assistance of films.

The Army roentgenologist must be acquainted with the normal procedures which pertain to procurement of x-ray equipment and materials and, thereafter, their proper handling. With most civilian institutional connections, the roentgenologist may sense merely a moral responsibility for protection of the equipment in his department. Unless he has actually purchased it, paying from his own resources, it is very natural that he should consider it the property of the institution, and it is very unlikely that unserviceability of any part of the apparatus would result in any financial loss to the doctor. Not so in the case of the Army roentgenologist! Though the equipment belongs to the United States Army, it is necessary that it be entrusted to a responsible person, an officer. In case proper accounting cannot be made for unserviceabilities or losses, a depreciated value of the equipment may be deducted from his pay.

Except during periods of actual combat, the Army roentgenologist must anticipate equipment and property needs. He must initiate the requisitioning of these. In this regard, he must realize the necessity of confining his wants, as far as possible, to a list of items considered most practical by our Supply Department. These items are contained in the supply catalog, which is ordinarily in the possession of the medical supply officer. The listing is of "standard items." A supply of such items is usually maintained at a near-by depot. Standard items

are listed according to classes. There are nine such classes. The classification has been made on the basis of general purposes or the type of the commodity.

The roentgenologist is mainly concerned with Class 6 or Class 9, insofar as x-ray equipments might be needed. Class 6 contains a listing of standard x-ray equipment such as might be needed for general hospitals and peacetime installations. Class 9 contains a similar listing of equipment which might be needed for installations such as the mobile surgical hospital or the evacuation hospital; installations operating in the theater of operations. Processing chemicals, such as would be needed for roentgenography, are contained in Class 1. For police and maintenance of a clinic, such as might be concerned with a fixed evacuation hospital or a general hospital, including furniture, cleaning items, stationery, miscellaneous office equipment and linens, are all listed under Class 7.

Nonstandard items are always more difficult to obtain, though where especially needed they can usually be obtained. They are not described in the supply catalog. Spare parts and replacements may be listed, though in many instances they, too, must be purchased as nonstandard items.

Items are carried as expendable or as nonexpendable. Furthermore, they may be identified as deteriorating or nondeteriorating. The roentgenologist must appreciate these characteristics and supervise the care and handling of this equipment as scrupulously as possible. In order to provide for efficiency in handling purchases and unit requisitions, it is necessary for him to understand the problems of the medical supply officer and to anticipate his needs so as to provide for including his requisitions as far as possible at prearranged times.

Except for expendable supplies, a central record is kept of all items issued. The roentgenologist is given a copy of the list for which he is responsible. It is important to inventory the property records and to identify each item when packing for a change of location, when reassembling and otherwise, at least once each month.

Illegal sales have been made of all types of Government property, but in particular, "consumption" of x-ray films must be watched. It is advisable that films be stored within a locked cabinet or a locked room, and that the keys to such be held by one person in the department. A tabulated record of quantities received versus quantities removed from this storage should be posted within this cabinet or room. This record should include the date, the film size, and the quantity concerned with each increment and with each issue, together with the initials of the person receiving them. This record need not be elaborate but it should be complete.

In larger x-ray clinics, besides keeping a record of all films received and all films issued, it is advisable that each roentgenographic technician and also those concerned with processing in the darkroom be required to report the total number of films used or handled each day or each week, as well as the number of films wasted. This system provides for checking the reports of the individual roentgenographic technicians with the summation report by the technician or technicians in the processing room, and both reports with the records contained in the film cabinet or film storage room. Usually, the psychologic effect of such a system will counteract any inclination toward "bootlegging" of x-ray films.

There is a similar temptation for certain people to enter into illegal sales of intensifying screens. These screens are very expensive. They can easily be removed from cassettes, and secondhand screens or blank cardboards may be used for substitutions. Because of such practices, it is advisable to identify each screen with the words: "Property of the United States Government." This description might be written with indelible ink along a margin of the screen whereby the wording will not be reproduced onto useful portions of the roentgenogram. If these captionings are accomplished in the handwriting of the radiologist, there will be provided a definite challenge in case roentgenograms are found in the department which do not bear his handwriting, even though it be evident that such roentgenograms were obtained with the use of cassettes.

These responsibilities pertain, in a general way, to all Army roentgenologists. Whereas one day he may be assigned to a task requiring knowledge in one phase of roentgenology, at another time he can expect a different or more comprehensive assignment. Anticipation of duty in the field of combat should be in the minds of all. In a previous paper[2] the responsibilities of the roentgenologist were considered in terms of the home communities versus the anticipated duties in the theater of operations. Preliminary work has been essentially concerned with chest examinations.[5] This phase, too, is subspecialized to the extent of having to deal with miniature films and with hundreds of studies each day—mass survey studies—and being guided by regulations with respect to acceptable versus unacceptable findings. Most of this work has been concerned with chest roentgenology—known to qualified roentgenologists and phthisiologists. The plan of this phase has been so well crystallized that detailed description of it would seem to be unimportant at this time. It must be realized, however, that these activities are continuing, and they must be expected again on a very large scale at a time of demobilization following completion of the gigantic task in which we are now involved.

SUMMARY

In general, it is realized that "roentgenology is roentgenology whether it be practiced in the Army or out of the Army." The Army roentgenologist is spared certain responsibilities, such as those concerned with obstetrics, gynecology, and to a large extent, those concerned with children's diseases and radiation therapy of malignancies. However, these exemptions are more than compensated by a number of requirements such as exceptional ingenuity, an ability to command men, and very diplomatically coordinate the roentgenologic service; particular acuity of diagnostic ability by way of roentgenoscopy without requiring film studies; familiarity with foreign-body localizations and familiarity with requisitioning and handling of equipment and supplies.

REFERENCES

1. de Lorimier, Alfred A.: Requirements for Roentgenological Services in the Field of Combat, Radiology 38: 590, 1942.
2. de Lorimier, Alfred A.: Wartime Military Roentgenology, Radiology 36: 391, 1941.
3. U. S. Government Printing Office, "Technical Manual 8-275," Superintendent of Documents, Washington, D. C.
4. de Lorimier, Alfred A.: Foreign Body Localization as Provided With United States Army Table Unit, Am. J. Roentgenol. 47: 307, 1942.
5. de Lorimier, Alfred A.: Mass Roentgenography of the Chest for the United States Army, Radiology 38: 462, 1942.

EFFECTS OF MODERN WARFARE ON CIVIL POPULATION

Experience of Great Britain*

D. Denny-Brown, M.B., F.R.C.P.

To begin with I shall outline some general aspects of air bombing and procedures taken by the civil authorities, such as evacuation and provision of shelters, because these affect the type and kind of nervous casualties from air raids. Indeed, prophylaxis is more important than treatment.

It was anticipated that any densely populated area which was subjected to military bombing would be the scene of mass panic and mass hysterical phenomena, as well as mass destruction. Yet, bombing of civilian population has failed to produce any sort of mass panic or nervous manifestations. That was first evident in Barcelona during the Spanish War, where the population settled down to a daily routine of their occupations for months of severe bombing. We have very little direct information of the effects in China or Warsaw or in Rotterdam, but there is certainly no report of such mass nervous reaction. There has been nothing of the sort in London or Coventry or any of the other British cities that have been intensely bombed. I think the chief reason is that the population is prepared by earlier, milder experience. If the Luftwaffe had taken the opportunity to bomb London on the very first day after war was declared and had caught a densely populated area at a time when nervous tension was at its height, the tale might have been different. But, in actual fact, there was sufficient warning, it was always obvious that things were getting worse, and nearly always people have experienced bombs at a distance before they have experience of bombs close by. Possibly most of those who feel they cannot go on with it manage to get away somewhere where bombing is not so intense.

The destructive effect of a bomb dropped from the air is extraordinarily limited. In the towns that have been bombed, you will see a house missing here, a shop missing there, or a large hole in the street. Houses on either side are battered by flying masonry but are not blown down. Within a radius of twenty or thirty yards there is utter destruction, and beyond that things are left standing. Within quite a much wider radius all glass, windowpanes, and often door frames, are broken by the blast of the explosion. Under those circumstances it is, therefore, fairly obvious that though a shelter of any sort has to be very strongly built, and if possible, to be deeply built in order to escape the effects of a direct hit, there are relatively very few chances of such a hit. A deep underground shelter is about the only place that is safe from a direct hit, but underground shelters are very expensive to build. It is impossible to build sufficient deep shelters for the whole population of a city. It would virtually be another city under the ground. Further, even deep shelters have

*Lecture XXIII, reprinted from *Collected Lectures*, the Metropolitan State Hospital, Waltham, Mass., 1942.

disadvantages in that occasionally an explosion near by will fracture deep water and gas conduits, and so bring danger of drowning or poisoning to the occupants. Many nervous people will literally spend all their time in deep shelters if they can. They are better evacuated from the area. It is more satisfactory to have people scattered as much as possible rather than have them all together in one place. The ordinary person is accordingly encouraged to take shelter in basements under or near his own home or near his place of work rather than to seek some large public shelter. A percentage of bombs dropped indiscriminately fall not in buildings but in streets, yards, and other spaces, even in a city. Any modern steel-frame building offers fair protection. Bombing of towns in broad daylight in the face of an air force that has any real strength has proved to be too expensive for the air force concerned, so that it is mainly a nocturnal experience. Shelters should be warmed, should be provided with some sort of sleeping accommodations, bunks, and they should be provided with adequate sanitation, and, if possible, with some first-aid arrangements.

While many people will take shelter, there is much to be done on the surface. The most efficient weapon of an air force seeking to destroy industry or communications is the incendiary bomb. Thousands of these bombs can be dropped rapidly in a small area, and twenty minutes later, when a few fires have started, another group of bombers can drop more high explosives and more incendiaries. If the incendiaries are extinguished, the damage is minimized and the bombers do not see their targets. It is essential to have large numbers of the population on the watch for them, on every roof with sand and spray pump to extinguish them rapidly wherever they fall. For this reason, in every area likely to be bombed the population has much to do besides take shelter. Clerks, bus drivers, professional men and women, all except children, have to take turns in fire-watching homes, industries, offices. Thus people are as widely dispersed as possible in any underground shelter or basement, and many others are fire-watching. In addition there is the ordinary force of civil air-raid wardens, ambulance and first-aid posts, and accessory fire tenders manned by part-time and full-time personnel, as well as the regular municipal hospital and fire services. One-third of the population is engaged in some form of "air-raid defense." In such a scattered and occupied population the casualty rate in one heavy night bombing in a place of 100,000 population would be approximately 250 to 500 people injured in the raid, depending upon its intensity.

A number of problems arise from this sort of organization. An air raid is a terrifying happening, but the effects are minimized by good organization. Hospital services are scattered as much as possible. Emergency light and other services are prepared. The sorting of casualties is arranged so that someone responsible sees every casualty on arrival and immediately attaches an appropriate label. The most important category is shock, because many patients with multiple injuries are dug out and are found to be severely shocked on arrival to the hospital. All patients referred with purely nervous symptoms are segregated. The responsibility on those who segregate the patient is high, for they should be able to distinguish shock and hysteria, be immediately able

to direct the excitable patient or the patient who is simply mute, or the patient who is in a state of stupor, to a place where he can be dealt with separately. It must not be forgotten that occasionally patients who are in a state of stupor may get very cold and shocked, even though they actually may not suffer any particular bodily injury. For many patients a hot drink and a sedative are sufficient. The most useful drugs are bromide and chloral, a mixture of 30 grains of bromide with 20 grains of chloral, but occasionally morphine is used. From 50 to 75 per cent of the patients suffering from nervous conditions during air raids will be ready to go home after having hot coffee and a sedative and after having been allowed to rest or sleep until morning. The remainder are admitted the next day to a subsidiary hospital outside the town. A large building in the country, a big school, or more often a mental hospital, part of which has been evacuated so that three of five or six wings would be used for the reception of casualties, is used.

With this sort of scheme, the original large city hospital is arranged to accommodate perhaps only 150 patients as a casualty hospital. In between air raids the normal population still attends out-patient clinics of the original hospital, and patients requiring hospital admission are admitted not to the parent hospital but to one of the rural subsidiaries. The arrangements are always such that the subsidiary keeps about three-quarters of the beds prepared for casualties. This system has never at any time been overstrained. In fact, there are many critics who say it is too elaborate. However, it is very possible that air raids will be more frightful still, since so far all belligerents have been spared the horrors of gas warfare.

The general procedure with nervous and psychiatric casualties is to move all who have not recovered in four to six weeks to special hospitals for such cases. It has been found necessary in London to have only two hospitals devoted entirely to nervous disorders, and neither of these has ever been completely filled. It has never happened that such a large number of strictly nervous casualties has occurred in any one place that they should have to be admitted direct to any particular hospital for nervous disorders. There were at the beginning at least three other special hospitals set up for nervous casualties, but these were long since disbanded. Most nervous casualties recover rapidly. By far the most common type of nervous casualty is stupor or hysterical fugue. When bombing goes on night after night for two weeks or more, a number of people break down, but usually before this condition occurs they are persuaded to go to the country for a one or two night rest, and are aided by some organization set up for the purpose. Everyone feels it is his or her duty to stay, so that some intelligent supervision is necessary to make the best of such arrangements. What most people need very badly is sleep, and a good organization will arrange beforehand to send everybody in rotation away from the city for two days with friends or billets in the country. They sleep solidly for the first twenty-four hours, then relax for another day and are ready to go back again.

Transient conditions of exhaustion, with anxiety symptoms and depression of various degrees, and fugues are extremely common. There are different kinds of dissociated state. In some the condition is very superficial, and the amnesia is recalled with only slight assistance. In others it is deeper and some

assistance by suggestion or hypnosis is required. I think it is essential to recover the amnesia in most cases. In still other cases the dissociated state or fugue is only one aspect of what is very much deeper emotional depression, and even if you succeed in clearing the fugue, a depressed state with retardation, often some depersonalization, remains. The illness has then a certain momentum which will take several weeks, perhaps five or six months, to overcome. Such patients must be dealt with in special hospitals with such treatments as continuous narcosis, which in my experience has been most satisfactory, except that it does require very skillful nursing and personnel. Convulsive therapy is little used for such cases.

It is difficult to get any statistics, for psychiatric casualties are mixed up with all others at the start, and are moved so rapidly and so far. No doubt official figures will be available in time. But I can say that from very large cities, such as London, the scale of persistent disability is very little more than the rate of peacetime nervous illness (their population being now much lower through evacuation of women, children, and some industries to the country).

Those dealing with psychiatric casualties have found, just as those dealing with psychiatric conditions in the Army and other services have found, that it is the person who has had constitutional history and previous bad personal history who is liable to break down. If you select those patients who have not recovered after six weeks, the proportion of them who have either bad family history, or history of previous breakdown, or both, is very high indeed, sometimes over 90 per cent. So it is the peacetime psychoneurotics that turn up under war conditions with breakdowns. People can adapt themselves to all conditions. If breakdown does occur, it has a natural tendency to recovery. Certainly I am sure young children are affected very much less than adults. Children up to the age of 3 or 4 do not appear to realize what is going on. I have seen nervous children between the ages of 6 and 12 years with tics, enuresis, troubled sleep, screaming attacks, tantrums, etc., if they are upset by an air raid, but most of them settle down very quickly. A very small percentage of children suffer from persistent nervous conditions, and these are usually constitutionally nervous children. In some all this disturbance of ordinary life is a help to psychoneurosis. Some chronic neurotics of peacetime now drive ambulances or fire tenders and complain no longer of their nervous symptoms. The more simple anxiety state, if there is something really worth while to get on with, can be really benefited by all this organization and feeling of urgency. If a breakdown occurs, it will, of course, be more severe. But the herd instinct can have beneficial effects, and the rejuvenation of the chronic psychoneurotic is one of them.

Apart from the direct effects of the air raid, there are other problems related to evacuation. It is important in densely populated areas to try to get everybody who is not occupied in essential work out to some other place, to move industry, and women and children from the densely populated areas to country areas where bombing is either not likely or not dangerous. Communications are not always good, and the wife with her children in the country soon gets worried as to what has happened to her husband in town. In England all evacuation is purely voluntary. The government pays the expenses of moving the family to the country, but, of course, the wage earner still has to pay the rent for his

apartment or house in town, and he has to contribute to the upkeep of the family in the country. There is a much greater stress on the family budget. Such anxieties can in part be dealt with by a good social service organization. A framework of experienced social service workers can function very efficiently if sufficient voluntary help is available from women's societies. A small bureau is established in every town and village for evacuated people, managed usually by one paid secretary and a number of volunteer workers, and is called the Citizens' Advice Bureau. Advice is given on all problems, communication with relatives, notification of casualties, procedure in relation to paying rents and claims for damages. Such an organization is an excellent prophylactic for psychoneurosis.

THE CHEMOTHERAPY OF BACILLARY DYSENTERY*

FURTHER OBSERVATIONS ON SULFAGUANIDINE

COMMANDER GEORGE M. LYON, M.C., U. S. N. R.

THE use of sulfaguanidine in the treatment of acute bacillary dysentery has been described in a report[1] based on the study of a small series of patients having the disease in a severe or moderately severe form. In this study alternate patients were treated with the chemical while others, as controls, were not. Since that time sulfaguanidine, the guanidine analogue of sulfapyridine, has had extensive practical application in the management of patients with "bloody flux" in the offices, homes, and hospitals of an area wherein bacillary dysentery is an important disease. An unusual opportunity for this study was presented by the occurrence, during this time, of what was probably, in the counties concerned, the highest incidence of the disease since 1926. More than 300 patients with "bloody flux" were treated with sulfaguanidine. Their records permit an appraisal of the effectiveness, and the suitability of this chemical agent for general practice in the treatment of acute bacillary dysentery, not only in the hospital, but of perhaps even more practical importance, in the offices and homes as well. A more detailed analysis of these records has already been presented.[2-4]

With few exceptions the method of administration employed was that originally recommended by Marshall[5] and employed in the previous clinical study.[1] The initial dose was 0.1 gram per kilogram of body weight, while the maintenance dose was 0.05 gram per kilogram of body weight administered orally every 4 hours until the stools were less than 5 in 24 hours, and then 0.05 grams per kilogram of body weight every 8 hours for 48 to 72 hours. That an adequate urinary output should be maintained, had been stressed repeatedly and in general was well observed. Particular attention was paid to the detection of any untoward effects which might result from the administration of the relatively new chemical.

*Received for publication, October 30, 1941.
Reprinted from the United States Naval Medical Bulletin 40: 601 (July), 1942.

From this experience certain impressions have been gained in addition to which there are some more definite conclusions which may be drawn, and it is, therefore, possible now to present further recommendations in regard to the clinical use of the drug.

Sulfaguanidine proved to be an effective therapeutic agent in the treatment of acute bacillary dysentery. When used in the manner described, and when treatment was begun during the first 5 days of illness, recovery usually occurred in 2 to 5 days. In the majority of instances, 5 days of chemotherapy was all that was required. When so used sulfaguanidine was entirely free from toxic effects. This freedom of dangers from toxic effects is in striking contrast to that of the other sulfonamide compounds, one of which, sulfathiazole, is described as being quite as effective as sulfaguanidine in the treatment of acute bacillary dysentery and which indeed may be more effective than sulfaguanidine in the treatment of the diarrheas resulting from parenteral infections.[6, 7]

As previously stated, when chemotherapy was begun within 5 days of the onset of fever or diarrhea, and when employed in the manner described, recovery almost invariably followed in 2 to 5 days. When not employed until later in the disease, results were frequently amazingly good, but failures were encountered somewhat more frequently. When treatment with the drug was begun after the fifth day and before the eleventh day, results, as a rule, were better than when begun after the eleventh day, but not as good as during the first 5 days. The importance of early treatment cannot be overemphasized.

When there was blood and pus in the stools and when there was fever present, even though the treatments were not begun early in the disease, results were nearly always as satisfactory as in the patients treated earlier. When, however, the dysentery bacilli had disappeared, and when there was no blood or pus in the stools; when the stage of bacterial activity was past and the stool was of the green watery type (so characteristic of the frequently encountered "post-infection" intestinal indigestion), sulfaguanidine had the least beneficial effects. This is to be expected, however, in view of the underlying pathology.

It is an outstanding characteristic of acute bacillary dysentery, although one frequently overlooked, that with remarkable frequency the stage of active bacterial infection is followed by an impaired ability on the part of the intestinal tract to digest and to assimilate food. It is generally accepted that this occurs as a result of the damage produced within the gastrointestinal tract by the dysentery bacilli, or their toxins. This form of "post-infection" intestinal indigestion was formerly very common among infants and young children, and is not infrequent today in those dysentery patients not receiving chemotherapy. Although less common in adults, this sequela is not at all an infrequent one. Since this condition results from damage done to the intestinal tract by the bacilli themselves, or by toxins produced by them, and since the bacilli by this time have usually left the intestinal tract, or have been supplanted by secondary invaders, one cannot expect sulfaguanidine to be such an effective agent in clearing up this diarrheal state. In actual practice it did not do so.

Sulfathiazole is said to be effective in those cases where the continued diarrhea is the result of secondary invaders, with or without deep ulceration. On

theoretical grounds there is indeed much to recommend it, or sulfadiazine, in such instances, and either may be more effective than sulfaguanidine. Fortunately, such conditions are not commonly encountered and practically never occur if chemotherapy is instituted early in the infection. Occasional cases of diarrhea were encountered which appeared to be simple watery diarrhea, without significant bacterial infection, without blood and pus in the stools, and which, despite adequate chemotherapy, did not yield to either sulfaguanidine or sulfathiazole. In such cases the diarrhea may possibly have been due to:

1. A previously undetected acute bacillary dysentery with the subseqnent development of the "post-infection" intestinal indigestion already described.
2. A parenteral infection not discovered.
3. A diarrhea due to a local or systemic virus infection.

In general, the best results with sulfaguanidine were obtained in those patients treated earliest after onset, who were most ill, and who had blood and pus in their diarrheal stools at the time chemotherapy was begun. It was least effective in those in whom treatment was not started until after the acute bacterial invasion was over, when the stools were simply green, watery, and without blood, pus, or dysentery bacilli.

Sulfaguanidine appears to be equally effective against all of the various strains of dysentery bacilli. Paradoxical as it may seem, it has appeared to have its most striking effect in the treatment of the toxic Flexner and Shiga strains. Two instances of Shiga were added and the results were the same as with Flexner. This has since been amply confirmed by Hardy and others. There is some limited evidence to suggest it was somewhat less effective against the clinically less toxic Sonne strain, one which so frequently gives rise to mild afebrile diarrhea without blood in the stools and often with little pus.

Most of the toxic effects which have been observed in the use of the other sulfonamides have also occurred following the use of sulfaguanidine. Such have, however, occurred almost invariably when the drug had been given in doses larger than recommended by Marshall and doses that were larger than those employed in the present study, and when, likewise, due regard was *not* paid to the importance of the frequency and the character of the stools, and to the relationship this has to the concentration of the drug in the intestinal tract when the stools are infrequent. Such toxic effects can be avoided almost entirely by employing the drug as recommended originally by Marshall, provided there is meanwhile an adequate urinary output, which definitely enhances the likelihood of there being no toxic effects. An adequate urinary output is always a desideratum in the treatment of acute bacillary dysentery. The fact that treatment is usually terminated in 5 to 7 days is an added safeguard against toxic effects. Toxic effects may have been encountered somewhat more frequently in adults than in infants or young children where they they were almost entirely missing.

Although sulfaguanidine was least effective after the first 5 to 10 days of illness, and after the blood and pus in the stools had given way to the plain watery stool which contained no dysentery bacilli, there is sufficient evidence at hand to indicate the desirability for the use of sulfaguanidine in all suspected cases of bacillary dysentery, "flux," or "infectious diarrhea" with

fever. It should be given for 5 to 7 days and thereafter if recovery has not occurred it may be best to give sulfathiazole, or still better, perhaps sulfadiazine, for another 5 to 7 days. In the meantime, strict regard must be paid to the urinary output, for with all of the sulfonamide compounds there is a strict relationship between adequate urinary output and freedom from toxicity. This is particularly to be remembered in the use of sulfathiazole and sulfadiazine.

Where there are obvious or suspected infections outside the gastrointestinal tract, it may be desirable, at the onset, to use sulfathiazole, or sulfadiazine, in the place of sulfaguanidine. While in such instances this practice may perhaps be more effective, in the experience described, it was never necessary to resort to it in the early stages of the acute infection. Also it presented no strikingly favorable results in the treatment of those cases with such complications observed 10 or more days after the onset of diarrhea.

Although potentially carrying a greater risk of toxicity, both sulfathiazole and sulfadiazine have been shown to be effective in the acute stages of bacillary dysentery. Because of their greater tendency to produce toxic effects, and because of the greater care with which they must be given, it would seem that they should be the drugs of secondary choice in the treatment of acute bacillary dysentery in the early stages. In the later stages, or in the state of "post-infection" intestinal indigestion, their use may be more strongly indicated. For use in the home, office, or dispensary they are definitely not so safe as sulfaguanidine, and should not be employed as sulfaguanidine may be. One of the most satisfactory features of sulfaguanidine is the large margin of safety permitted in this respect without sacrificing therapeutic efficacy. It is necessary, frequently, to treat cases of acute bacillary dysentery without the benefit of hospitalization. For them sulfaguanidine is distinctly the drug of choice. Experience has proved that good results have followed this practice.

Excepting newborn, no deaths occurred among the patients treated with sulfaguanidine, provided treatment was begun during the first 5 days of illness (diarrhea and fever) and such cases did not have other important infection outside the intestinal tract. Parenteral infections, whether due to a virus of the influenza type, or to pyogenic bacteria, were observed to exert a detrimental influence on the clinical progress of the patient with bacillary dysentery just as they have on other diarrheal states. It is not logical to expect that such parenteral infections would yield to sulfaguanidine therapy. On the other hand, it was observed, more frequently than not, that under the influence of the drug, the stools became normal promptly and the intestinal tract appeared better able to carry out its work.

In the latter stages of acute bacillary dysentery or its sequela, it may be impossible to determine which of the difficulties encountered are due to a continuation of the primary bacterial invasion, to secondary invaders of the intestinal tract, to parenteral infection, or to damages within the intestinal tract due to the primary bacterial invasion. This complicates the problem of appraising the effectiveness of any chemotherapeutic agent used in acute bacillary dysentery or other diarrheal states.

No persistent states of "post-infection" intestinal indigestion developed in any of the patients in whom chemotherapy was started within 5 days of the onset of fever or diarrhea.

Those cases receiving the chemical early showed surprisingly little disturbance of digestion either during the period of active therapy or following recovery. In spite of this, however, it has seemed advisable to employ a bland diet, moderately restricted in amount, during the period of active treatment. There is some reason, based upon careful observation, to believe that those patients who were on a diet which was high in protein (casein), and which was moderately restricted in amount, presented the best and most prompt recoveries. Cultured lactic acid milk, cottage cheese, Philadelphia cream cheese, and custard, when given in moderate amounts, seemed to be the diet of choice. Vitamins, in powder form, were added to the cultured lactic acid milk and may, or may not, have influenced the recoveries.

Sulfaguanidine was most effective during the time when the dysentery bacilli were more active. It was least effective when the activity of these bacteria was absent and when their residua remained within the intestinal tract in the form of abnormal physiologic states. While the chemical effectively controlled the activity of the bacteria, it did not, by itself, correct damages already produced by the bacteria, or their toxins, once such damage was already established. By controlling the bacterial infection, sulfaguanidine may have aided such supportive measures as adequate fluid intake, intravenous glucose, calcium gluconate, transfusions of serum or whole blood, etc., so that they might, more effectively, correct attendant chemical pathology. It should be emphasized that sulfaguanidine did not and will not overcome acidosis, dehydration, fluid imbalance, or electrolyte disturbance.

When altered physiologic states, or those characterized by unusual chemical pathology, were already present before the institution of chemotherapy, it was necessary to employ measures directed toward these specific conditions. In the more protracted forms, and in the absence of such special measures, chemotherapy would undoubtedly have been much less effective.

Sulfaguanidine was not as effective in recurrent attacks of bacillary dysentery, nor in the more chronic forms, as it was when used within 5 days after the onset of the original attack. If, on the other hand, the stools contained blood or pus, and there were dysentery bacilli in the stools as shown by culture, the results were generally very good. It was difficult to predict what the result would be. At times, so striking and so prompt were the beneficial effects of the drug, in these forms of bacillary dysentery, that one cannot escape the conviction that sulfaguanidine should be administered in all forms of bacillary dysentery for at least 5 to 7 days in the manner recommended. If at the end of that time recovery is not apparent, then sulfathiazole or sulfadiazine should be given for 5 to 7 days. Some of the most amazing results so far encountered in the use of sulfaguanidine occurred in stubborn, serious cases of chronic bacillary dysentery.

Relapses, whether early or delayed, were extremely rare after the use of sulfaguanidine in the treatment of acute bacillary dysentery. Certainly this was in striking contrast to the frequency with which such conditions occurred in those who did not receive chemotherapy.

There was no reason to believe, either from the bacteriologic or from the epidemiologic standpoint, that the use of sulfaguanidine tended to "mask" infections of dysentery bacilli, or to complicate the community health situation by increasing the "carrier" rate.

In homes where outbreaks resembling the institution type of outbreak of bacillary dysentery occurred, it was possible, with sulfaguanidine, to control promptly and effectively, not only the individual cases, but also the spread of the outbreak as well. The chemical has great value as a control agent in outbreaks occurring among those whose living conditions are crowded, which so frequently are encountered in mining areas, in the poorer districts, and even in military camps and establishments. It is possible in an institution to check such an outbreak within a few days by giving everyone in the institution the chemical for a period of 5 to 7 days. It must be remembered that in so doing the maintenance dose should be employed and given only every 8 hours if the stools are less than 5 a day as they are apt to be in those not already stricken with the malady.

Sulfaguanidine is of definite value in reducing the number of days of illness due to bacillary dysentery. This gives it unusual importance in industrial and military medicine. Its wider use will result in a continuously lower dysentery mortality rate.

Early recognition and early treatment are just as important in the treatment of bacillary dysentery as the antibacterial activity of the chemical itself. Best results were definitely attained when chemotherapy was applied early in the disease.

Sulfaguanidine is quite as effective in the treatment of acute bacillary dysentery in adults as it is in infants and young children.

Chemotherapy has completely revolutionized the treatment of bacillary dysentery. Sulfaguanidine is quite as effective in the treatment of acute bacillary dysentery as sulfanilamide is in the treatment of some streptococcal infections or as the other sulfonamide compounds are in the pneumococcal infections. Besides its therapeutic effectiveness, the chemical is easy to give and has such a wide margin of safety that it is the ideal agent to use in the treatment of bacillary dysentery.

NOTE.—Since submitting this report, it has been possible to observe the treatment of 14 patients with severe or moderately severe bacillary dysentery, who were treated with succinyl sulfathiazole. In severity and in clinical characteristics these were in all respects similar to those previously described in a controlled study.[3] The initial dose was twice the maintenance dose. This in turn was 0.04 gram per kilogram of body weight every 4 hours if six doses were given daily, or 0.05 gram per kilogram if five doses were given daily at approximately 4-hour intervals. After the diarrhea had been "checked" for 72 hours the drug was discontinued. In this admittedly small series of cases, succinyl sulfathiazole appeared to have all the virtues of sulfaguanidine as to therapeutic efficacy and as to freedom from untoward effects. It was exceedingly well tolerated.—[G. M. L.]

BIBLIOGRAPHY

1. Lyon, G. M.: Sulfanilylguanidine; Treatment of Acute Bacillary Dysentery, U. S. Nav. M. Bull. 39: 278-293, April, 1941.
2. Lyon, G. M.: Round Table Discussion on Chemotherapy, Annual Meeting, American Academy of Pediatrics, Boston, October 9, 1941, J. Pediat. 21: 809, 1942.
3. Lyon, G. M.: Symposium on Chemotherapy. Section on Medicine, Annual Meeting, Southern Med. Assn., St. Louis, November 13, 1941. (In press, South M. J., May, 1942.)
4. Lyon, G. M., Folsom, T. G., Parsons, W. J., and Sprouse, Irma: Sulfaguanidine in the Treatment of "Bloody Flux," W. Va. Med. J. 38: 19-27, January, 1942.
5. Marshall, E. K., Jr., Bratton, A. C., Edwards, L. B., and Walker, E.: Sulfaguanidine in the Treatment of Acute Bacillary Dysentery in Children, Bull. Johns Hopkins Hosp. 68: 94-111, January, 1941.
6. Ravenel, S. F., and Smith, D. L., Sr.: Chemotherapy of Bacillary Dysentery, South. M. J. 38: 504, May, 1941.
7. Davison, W. C., and Casparis, H.: Personal communications.

MEDICAL ILLUSTRATION IN THE UNITED STATES ARMY*

Historical and Present Considerations

Captain Ralph P. Creer,† U. S. Army

It has been said that the only worthwhile result of any war is the great advancement that is produced in medical science. If this is true, then it follows that proper and adequate illustrations of these achievements are important.

Medical illustration in time of war is by no means new. The ancient Greeks and Romans produced illustrations of a medicomilitary nature (Fig. 1). As early as 1497 line drawings appeared in *The Book of Surgery*. One illustration entitled "The Wound Man" shows many types of injuries by various weapons and external agents, such as those acquired from a thorn, an arrow, a club, a knife, a sword, and similar devices (Fig. 2*A*). Another woodcut published in 1528 illustrates the disinfection of a wound by cauterization (Fig. 2*B*). The extraction of an arrow from the cardiac region by military surgeons was also depicted in 1528 (Fig. 2*C*).[2]

The surgical work of Ambroise Paré,[3] famous French surgeon, published in 1634, contains medical drawings. The *Memoirs* of Larrey,[4] surgeon to Napoleon, is illustrated with fine steel engravings of war wounds and instruments.

There are many other examples of early medicomilitary art. However, in the interest of brevity this paper is limited to medical art in the Army of the United States, beginning with the Civil War.

On Nov. 24, 1863 Gen. Joseph K. Barnes, Acting Surgeon of the Army, issued the following Circular Letter No. 26:

> The attention of Medical Officers in charge of U. S. A. General Hospitals is invited to the importance of preparing illustrations of the results of surgical operations. These can in many instances be conveniently obtained by means of plaster casts, which are readily made without subjecting patients to the slightest inconvenience.
>
> The casts most desired are those of stumps of amputations of every variety, and models of limbs upon which excisions may have been performed.

Soon after the War Between the States, *The Medical and Surgical History of the War of the Rebellion*[5] was published. This group of stately volumes gave

*From the Army Medical Museum, Washington, D. C.
†Sanitary Corps.
Received for publication, January 5, 1943.

a clear visual idea of the type of wounds encountered, the means of surgical repair, and the many traumatic diseases which then as now were problems of all wars.

There were a number of contributors to these well-illustrated books. Of course, the early photomicrographic achievements of Col. J. J. Woodard, Major Curtis, and W. M. Gray are well known to students of photomicrography. William Bell, Charles Throught, and C. J. Blacklidge made most of the clinical and gross pathologic photographs.

The drawings were contributed by Baumgrass, Dr. J. C. McConnell, William Schultze, Edward Stauch, and Hermann Faber. The latter had a distinguished career as an artist. At the outbreak of the Civil War he enlisted and was assigned to the Surgeon General's Office as a medical artist, although rated as a hospital steward. He remained in the Service for five years and after the war returned to Philadelphia, where he continued to practice his profession. Together with his two sons, Ludwig and Erwin, and Louis Schmidt, he illustrated Piersol's *Human Anatomy*[6] and other important texts.

Special mention should also be made of Gen. George M. Sternberg, who served as a medical officer in the Civil War at the age of twenty-three. Bacteriology and photomicrography were his main interests and in his biography published in 1920 by his wife Martha[7] the following statement is found: "After our arrival at Fort Mason, Dr. Sternberg was not long in establishing at his own expense a laboratory for biologic research. It was here in 1881 that he demonstrated and photographed the tubercle bacillus, discovered by Professor Koch earlier in the same year. I am informed on good authority that this was the first demonstration of the organism in America."

He was the author of a textbook entitled *Photomicrographs and How to Make Them.*[8] In 1879 Sternberg published a well-illustrated atlas of photomicrographs. He later became Surgeon General of the United States Army.

Medical illustration in the United States Army received its greatest impetus during World War I. In June, 1917, Mr. Roy Reeve was assigned as photographer at the Army Medical Museum. Almost immediately the need for a more active service was recognized and in less than a year the personnel of this department was increased to approximately twenty. Their duties for the most part consisted of photostat copying, and a number of lantern slides were prepared for teaching purposes. This was the beginning of a permanent photographic department which has contributed ever since to the effective and efficient dissemination of medical knowledge.

In the same year, 1917, a medical art department was organized under the supervision of Lieut. William T. Schwarz and Lieut. M. L. Bauer; a motion picture section under the direction of Lieut. Thomas Evans also was started.[9] The function of the art department is best described in an article by Major R. W. Schufeldt published in July, 1918.[10]

> The scope of the Art Department was, when first put on foot, very modest, being principally confined to the making of a series of drawings and designs for use in motion pictures. Then man after man was added, each skilled in some special line, until at this writing there are first-

class painters in both oil and water colors; anatomical and pathological artists, histological artists, as well as modelers and casters, designers—indeed, technical artisans of nearly every sort known to the professions and trades.

Another field into which the output from this art department finds its way is the instruction of men and officers at the various cantonments and camps, in this country as well as in France. This is accomplished through the scientific use of the motion picture machine and the lantern.

Fig. 1.—The physician Iapyx extracting an arrowhead from the thigh of the wounded Trojan hero Aeneas. From a Pompeiian wall painting.

Competent medical officers, who are good instructors, are sent out on this work, equipped with projecting instrument, screen, and films or slides, and give a complete set of lectures at the camp or cantonment to which they are directed to proceed. This entire scheme has resulted in vastly improving the fitness and health of both officers and men of our army, and the amount of improvement so produced is now being very rapidly increased. Our art department has very substantially aided in this, and its assistance is being felt more and more each day that passes.

Most of the artists assigned were portrait or commercial painters with little or no knowledge of medical illustration. Therefore, a complete dissecting room was provided where these men made actual dissections upon a cadaver under the supervision of a competent anatomist and teacher.

The motion picture department was charged with the responsibility of producing instructive teaching films in relation to health for Army consumption. The original negatives were made by this department and a number of duplicate films were processed by the personnel. The motion picture "Fit to Fight" is an example of the type of film produced by the department; nineteen copies were distributed throughout the Army camps. Other two-reel anatomic pictures relating to veneral diseases were also made.

During the course of and directly following the last war two exceptionally worth-while texts were published. In each instance medical illustrations were of incalculable value.

A Manual of Surgical Anatomy[11] was authorized by the Secretary of War. Supervised by the Surgeon General, this manual was prepared at the University of Illinois and the illustrative work was executed under the direction of Mr. Tom Jones.

In 1920 a book entitled *Studies on Pathology of War Gas Poisoning* was published by Dr. Milton Winternitz. The work was done with the consent of the Surgeon General of the Army and the Chemical Warfare Service. This volume was well illustrated by Mr. Armin Hemberger, a medical artist at the Yale University School of Medicine.

Early in April, 1918, Col. Louis B. Wilson reported to France for duty as Director of the National Army Medical Museum Unit. Soon after his arrival he visualized the need for a competent medical illustration service and in May, 1918, a request was made for clinical photographers and artists who were already on duty at the Army Medical Museum in Washington. This request was disapproved by the general staff "in view of the existing tonnage situation, and in the belief that the requirements could be successfully handled by the Signal or Engineering Corps."

An attempt to locate qualified personnel in these organizations proved futile. Thus, a second request was made to the War Department and finally on Sept. 14, 1918, one officer and seven enlisted men arrived in France prepared to make motion pictures of war wounds and injuries. A few weeks later several medical artists and moulage experts reported for duty and a general illustration unit was established at Vichy in the central hospital laboratory of the Base Hospital Center.

Prior to the arrival of this last group a number of units had brought with them fairly good amateur photographers. However, in many instances these men were put into work entirely foreign to photography and considerable time was lost in effecting their transfer to departments where their photographic ability would be available to the medical service. Furthermore, Colonel Wilson encountered some difficulty in arousing interest in medical motion pictures. Most of the surgeons at that time did not see any value in motion pictures of surgical operations, and this particular branch of medical illustration was not encouraged.

Furthermore, General Orders No. 15, H., A. E. F., Jan. 24, 1918, limited the practice of photography in the American Expeditionary Forces to the Signal Corps. On May 25, 1918, another General Order was issued which amended the previous orders and charged the medical department with making

A.

B.

Abb. 91. Ausbrennen einer Wunde mit einem Glüheisen. Holzschnitt in der Weise Wechtlin's aus: H. v. Gersdorf, Feldbuch der Wundarzney. Straßburg, Joh. Schott, 1528.

C.

D.

Fig. 2.—A, The "wound man" from Hieronymus, 1497. B, A woodcut, probably by Wechtlin, showing disinfection of wound by cauterization, 1528. C, The extraction of an arrow from the cardiac region by military surgeons, 1528. D, Woodcuts illustrating the treatment of fractures of the knee, thigh, and arm, 1528.

technical photographs of surgical and pathologic interest. In order to carry out this responsibility a survey of the medical department was made to ascertain the availability of suitable photographic equipment.

Because of the previous ban on photography by the Medical Department practically no hospitals were found equipped with sufficient apparatus or materials. A few cameras were procured from French sources, three were borrowed from the Signal Corps, and twenty-four from the roentgenologic department of the professional service. When finally equipped this group produced thirty-five casts of surgical cases, about 200 drawings, and more than 1,000 photographs of technical subjects prior to the signing of the armistice of Nov. 11, 1918, approximately three months of actual service.

Although the Signal Corps had been authorized in March, 1918, to illustrate the medical history of the war, they had been able to cover but little of the medical activities of the American Expeditionary Force, except for popular subjects which were used for propaganda purposes. After the signing of the armistice, and as soon as the clinical photographers could be released from their duties with combat divisions, they cooperated with the Signal Corps in illustrating the medical history with still and motion pictures. This bureau made more than 10,000 still pictures and about 40,000 feet of motion pictures of hospital activities, hospital locations, and group and individual pictures of the medical officers of the General Staff.

As a result of his knowledge of the organization difficulties which were encountered during the First World War, Colonel Wilson wrote in the February, 1920, issue of the *Military Surgeon*[13] as follows: "Restrictions on photography, such as operated early in the history of the A. E. F. so greatly to hamper the activities of the Medical Department, would seem to be unnecessary for safeguarding information, the Laboratory Division no doubt being as trustworthy as the Signal Corps. Preparation should be made in peace time for the selection of trained personnel for the various highly specialized functions which must necessarily be performed. Provisions should also be made for a standard photographic equipment to be supplied to the laboratories of various types. Special attention should be paid to photographs of methods of handling the wounded and of the appearance and treatment of battle injuries in forward areas, an adequate knowledge of the conditions of which is so essential and yet so difficult to convey to the inexperienced medical officer."

Similar ideas were suggested and the value of medical illustration in time of war was outlined by the author in a paper presented at the Annual Convention of the Association of Military Surgeons in October, 1938.[14]

At the request of the Surgeon General's Office the Biological Photographic Association in 1940 furnished a list of competent and qualified clinical photographers. Mr. Tom Jones and Mr. Jack Wilson cooperated in supplying a similar list of medical artists.

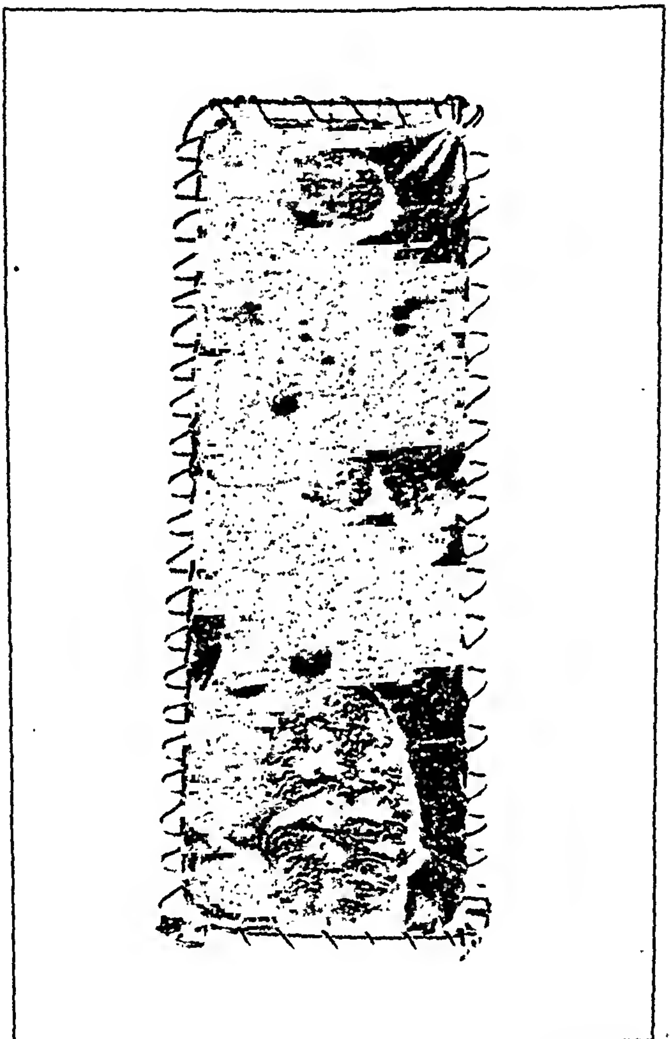
Early in 1942 Col. J. E. Ash, Curator of the Army Medical Museum, was charged with the responsibility of organizing the "Museum and Medical Arts Service." The purpose of this newly formed unit is to provide an adequate

A.

B.

Fig. 3.—*A*, Shot wound of the thorax and abdomen, with hernia of the lung. A reproduction from the original drawing by Edward Stauch. (*Medical and Surgical History of the War of the Rebellion*, Part I, Vol. II.) *B*, Gunshot fracture of the right acetabulum and the head of the femur. Reproduced from a lithograph of an original photograph. (*Medical and Surgical History of the War of the Rebellion.*)

and efficient illustration service for the Medical Corps. This new organization is attached to the Museum for training purposes and all material collected by such units will be shipped to the Museum, where it will be available for study and research.



THICKENED PEYER'S PATCHES.

No. 380 MEDICAL SECTION.

Fig. 4.—Photograph of a gross specimen. (*Medical and Surgical History of the War of the Rebellion.*)

In addition to the illustration activity this organization will also be responsible for the collection of missiles and helmets that have been struck by gun or shell fire, of medical and surgical instruments of American, allied or enemy origin, especially those which have been developed or materially

modified during the progress of the war, and of any other similar apparatus that might prove of historical value. Pathologic specimens are also to be included in the collection.

Units will be attached to the larger medical installations in the various important theaters of operations and will be under the direction of the surgeon of each theater. It is highly probable, therefore, that a unit may be temporarily divided and a photographer or artist ordered to cover an individual assignment.

Provisions have also been made for the employment of a limited number of Civil Service personnel. These men or women will be attached to the larger general hospitals within the continental limits of the United States and will be assigned wherever their services are required.

These units will be equipped with still and motion picture equipment, together with material to produce color as well as black-and-white photographs. Suitable and adequate art supplies will be furnished to the medical artists. All of the photographic supplies and most of the art materials will be furnished by the Signal Corps while in the theater of operations.

Arrangements have been made whereby the Signal Corps facilities will be available for photographic processing. Proper labeling and the identification of all illustration material is of the utmost importance, and each man is specifically instructed in this regard.

The qualifications of the officers in charge of each unit will be of special interest, and the following is a brief, concise outline of the requirements.

1. It is essential that the officers have a rather broad training with experience in the field of medical illustration, particularly photography.

2. They must be experienced in supervising, planning and directing the activities of a medical illustration department.

3. According to The Adjutant General's Office, applicants for a First Lieutenant in the Sanitary Corps must be at least thirty years of age, have a college degree, and, of course, there is always the matter of an Army physical examination.

4. Last, but by no means least, the candidates must be *officer material.* In that phrase is embodied a number of qualities which are quite aside from the professional requirements, but are extremely important to the success of an officer and his entire unit of men.

With regard to the enlisted men, thus far we have been able to select men who have served as assistants in various medical photographic and art departments throughout the country. Most of them were already in the armed service prior to the organization of the Museum and Medical Arts Service, and it was necessary to effect a transfer so that their special training would be available to the Medical Department. In other instances, they have been enlisted for duty at the Army Medical Museum.

In any event, it is essential that each enlisted man receive the required three months' basic military training at an Army camp.

The same requirements hold true for the artists. The majority of the artists now on duty have had formal training in medical art. A great many portrait and landscape artists have applied in writing or in person. One of our

greatest problems is the matter of explaining the fact that medical illustration requires a special type of training decidedly separate and distinct from the usual academic art course.

At present the artists on duty at the Army Medical Museum are undergoing a training program especially designed to help those who require further instruction in surgical drawing and moulage. They are also preparing basic anatomic drawings for the surgical service of the Surgeon General's Office. In addition, these men prepare drawings of unusual gross specimens which are shipped to the Museum from various Army medical centers. Their cooperation is also solicited in the preparation of scientific exhibits.

The photographers receive training and actual experience with the apparatus and materials which will be employed in an overseas assignment. The photography of patients, gross specimens, and surgical operations will account for the greater portion of their work. There will also be a limited amount of photomicrography.

Although the professional relationship between the medical artist and clinical photographer has shown a decided change for the better in recent years, nevertheless there is still much room for improvement. The majority of enlisted personnel attached to the Museum and Medical Arts Service are younger men. It is necessary that they drill together, work together, often live in the same barracks, and enjoy the same recreational facilities. As a result, it is only logical to assume that in the future men in this profession will enjoy a better understanding and appreciation, and a keener respect for each other's specialty.

Inasmuch as both officers and enlisted men will eventually be assigned to military hospitals or field units, a military training program is essential. This program consists of lectures, training films, and drill formation.

The Training Division of the Surgeon General's Office is working on a plan whereby realistic full-size wax models of various war injuries will be used to demonstrate the actual appearance of war wounds to the enlisted men of the Medical Corps. In addition, duplicate casts will be prepared of latex or a suitable substitute. These thin, sleeve-like appliances, molded and colored to simulate actual war injuries, can be affixed to the arm, leg, or other parts of the body. Worn by men on maneuvers they will convey a realistic sight of what may be encountered on the scene of action. Dressings, splints, and tourniquets may be applied and later checked by the Medical Department instructors.

From the foregoing it can be concluded that the Medical Corps is fully aware of the importance of medical illustration, especially in time of war. Careful preparation and planning have been accomplished, and we are now ready with suitable equipment and trained personnel to meet the illustration needs of the Medical Corps. Obviously this entire program places in the hands of the Medical Department the same powerful documentary and educational implement of visual description which is rapidly revolutionizing the entire teaching profession.

REFERENCES

1. Brunschwig, Hieronymus: Chirurgia. (German) Strassburg, Johann (Reinhard) Gruninger, July, 1497.
2. Gersdorff, Hans von: Feldtbuch der Wundarznei, Strassburg, Schott, 1528.
3. Paré, Ambroise: The workes of that famous Chirurgion, Ambrose Parey, Transl. out of the Latine and compared with the French by Th. Johnson. London, T. Cotes & R. Young, 1634.
4. Larrey, De D. J.: Memoires de Chirurgie Militaire, et Campagnes Tome I, Paris, 1812.
5. United States War Department, Medical Department. The Medical and Surgical History of the War of the Rebellion (1861-5). . . . Washington, 1870-83.
6. Piersol, George A.: Human Anatomy, Including Structure and Development and Practical Considerations, J. B. Lippincott Co., Philadelphia, 1907.
7. Sternberg, Martha L.: George Miller Sternberg, A Biography, Chicago, 1920.
8. Sternberg, George M.: Photo-micrographs and How to Make Them, Boston, 1883.
9. Shufeldt, R. W.: Activities Incident to the War, as Seen at the Army Medical Museum in Washington. Med. Rev. of Rev. 24: 137, 1918.
10. Shufeldt, R. W.: The Art Department of the Army Medical Museum, Med. Rev. of Rev. 24: 390, 1918.
11. Manual of Surgical Anatomy, Authorized by the Secretary of War under the Supervision of the Surgeon General and Council of National Defense. 1918.
12. Collected Studies on the Pathology of War Gas Poisoning, M. C. Winternitz, Yale University Press, New Haven, 1920.
13. Wilson, Louis B.: Museum and Art Service of the American Expeditionary Forces, The Military Surgeon 46: 165, 1920.
14. Creer, Ralph P.: Clinical Photography in the United States Army, The Military Surgeon 84: 232, 1939.

WAR AND DISEASE

RALPH H. MAJOR, M.D., KANSAS CITY, KAN.

DISEASE profoundly influences the course of men's lives. Man, from the day of his birth to the day of his death, is in constant battle against disease, either warding it off or trying to conquer it. Disease has determined the migration of races, their colonial expansion, their geographical distribution. It has conquered armies, overthrown empires, and changed the course of history.

In times of peace, as well as war, great epidemics have left their mark upon the history of nations. The fall of the Venetian Republic was caused as much by the constant incursion of bubonic plague as by the armed attacks of her warlike neighbors. The Black Death in England produced as great a revolution in the social and economic life of Britain as all the wars that hardy island waged. The terrific toll the plague took among priests and higher ecclesiastics necessitated the filling of these depleted ranks by ill-trained and often ignorant peasants. Many historians have considered that these clerics, possessed of little respect for the prerogatives of the clergy and more concern for the lot of the common people, were among the most powerful influences leading to the English Reformation.

Dramatic as are the changes produced by disease during times of peace, the effects of disease in times of war are even more cataclysmal. Wars spread disease, and disease, in turn, destroys warriors. Epidemics in past wars have only too often destroyed armies and left nothing behind but a melancholy

account of their progress. In some wars, epidemics, although they have destroyed thousands, have left some clue that enables later generations to conquer or to prevent them. In other wars we have the melancholy picture of the deaths to thousands which could have been prevented by the intelligent application of measures already known to medical science. This picture, unfortunately, appears with distressing frequency as we turn the pages of medical history.

The great plague of Athens was one of the most dramatic episodes in the history of civilization. Whether this was a bubonic plague, as Francis Adams of Banchory, who was not only an eminent physician but an outstanding Greek scholar, asserted, or whether it was typhus fever or scarlet fever as others maintain, is beside the point. The Athens of Pericles presented a level of culture and intelligence never before or since equalled. Within the walls of this city of some two hundred thousand, lived and worked the dramatists Aeschylus, Sophocles, Euripides, and Aristophanes; the philosophers Plato, Xenophon, and Anaxagoras; the sculptors Phidias, Praxiteles, and Polygnotus, and the historian Thucydides. When the Spartan king Archidamus attacked Athens, Pericles followed what is called today a "scorched earth" policy. He withdrew all his soldiers and people from the outlying country and they swarmed into the walled city of Athens for protection. The housing conditions were inadequate, and the food supply was insufficient; the proper disposal of refuse became impossible, and the water supply became contaminated. Then, like a thunderbolt, the plague appeared with all its horrors which have been so graphically described by Thucydides, an eye witness of the harrowing scenes.

The Spartan king prudently withdrew and attacked elsewhere. He feared the epidemic might spread into his own army, and he also wisely reckoned that the plague would destroy more Athenians than could the Spartans. According to some authorities, the first wave of the epidemic destroyed one-third of the inhabitants of Athens; according to others, two-thirds. After the first wave had subsided somewhat, Pericles sent an expedition to capture Potidaea and harass the Spartans from the rear. This expedition, if successful, would have changed the entire course of the war. Unfortunately, the men carried the seeds of the pestilence with them and, before the ships reached Potidaea, the disease raged among the soldiers and sailors, and the fleet was compelled to return to Athens. Pericles then planned another brilliant stroke. He sailed with his troops to Epidaurus, hoping to capture that city and again strike the Spartans from the rear. This expedition was also a dismal failure because, according to Plutarch, "the distemper which prevailed in his army broke all his measures, for it not only carried off his own men, but all that had intercourse with them."

Athens slowly went to her doom. When the plague struck down Pericles himself, no leader of equal ability arose to take his place. Finally, the Spartan admiral, Lysander, sailed into the harbor of Piraeus; the Spartans tore down the long walls of Athens to the merry tune of Spartan flutes, and young Spartan women danced gaily to the music while the Athenians filled the air with wails and lamentations. The Athenian Empire was humbled to the dust, never to rise again. The plague of Athens had played a catastrophic part in her defeat, and had left no clue to prevent similar catastrophes.

Unfortunately, the historians of the Peloponnesian War—the war between Athens and Sparta—were not medical men. If Hippocrates, as well as Thucydides, had described the plague of Athens, there would probably be no uncertainty concerning the nature of this epidemic. Fortunately, some of the epidemics accompanying later wars were described by surgeons in varies armies with such accuracy and fidelity that the diagnosis is certain.

Few wars in history have lasted so long or have been more destructive of life and property than the Thirty Years' War. This war, which began as a struggle between Protestants and Catholics, became a war in which the Protestants of Germany and Sweden were allied with Richelieu, the great Catholic prelate, in the struggle against Catholic Austria. This war was a "total war" in the modern sense. Far more civilians—men, women, and children—were destroyed than soldiers. Germany, according to some authorities, lost one-half of her entire population; according to others, two-thirds. A century and a half passed before the population of Germany reached its pre-war level.

The Thirty Years' War is commonly divided by historians into the Bohemian period, the Palatine period, the Danish period, the Swedish period, and the French period. The medical historian, Prinzing, divides it into the typhus period and the plague period.

The disease which carried off so many victims in the early part of this war was the notorious Hungarian disease, a disease accurately described by Tobias Cober, who served as surgeon in the army of Rudolf II, King of Hungary, in the wars against the Turks. He notes that the disease raged especially in camps heavily infested with lice, and his description leaves no doubt that it was typhus fever.

Measures were taken by certain cities to prevent the twin pestilences, typhus and plague, from entering. In Munich, for instance, the city fathers shut the city's gates against all strangers, disinfected all incoming letters, and washed with vinegar all the money that came in. These measures stayed the hand of the plague for a time but presently, infected Spanish soldiers forced their way into the city and spread the disease among the inhabitants. More than one-half the population died from the plague in a few months.

Many great generals, Tilly, Wallenstein, and Gustavus Adolphus, perished in the course of the war. When we balance the results of this war in the terms of great victories, territory gained, and prestige enhanced, as against the losses through pestilence, we may safely conclude that the real victors were typhus and plague.

Napoleon, the greatest military genius of the nineteenth century, was really defeated before Waterloo; he was defeated by his ill-starred campaign in Russia. The Grand Army, to quote Larrey, Napoleon's great surgeon, set out for Russia 400,000 strong. Six months later this army, retreating from Russia, finally reached Gumbinnen in East Prussia, numbering only 3,000.

This disastrous adventure of Napoleon has been described in every history of Europe. The bloody battles, the intense cold, the ferocity of the Russian Cossacks, have all been stressed as the cause of Napoleon's defeat. The role played by disease was even more important. Typhus fever seems to have played the major role, ably assisted by typhoid and dysentery.

The accounts left by the surgeons in Napoleon's army give us a clear picture of the devastation caused by disease. One of the most dramatic was written by Kerkhoffs, a Dutch surgeon attached to the Third Army Corps of Marshall Ney. This Corps, Kerkhoffs relates, was finally reduced to twenty men, as the result of privation and disease. In discussing the failure of the campaign, Kerkoffs remarked that if Napoleon had contented himself with occupying Poland and organizing sanitation there, the campaign would have succeeded and changed the whole course of history.

But Napoleon was in a hurry. In spite of bloody battles and ever-increasing number of sick, he pressed on to Moscow, carrying wagon loads of infected soldiers into that city, leaving the desperately sick in towns and villages where they infected the new troops pressing on eastward.

Sergeant Bourgogne of the Grand Army wrote an interesting account of the march into Russia. He was particularly impressed by the large number of vermin he saw. He went to sleep on a reed mat in a little town of Poland. Presently he awoke from his slumber and finding himself covered with lice, stripped off his shirt and trousers and threw them into the fire. There were so many of these animals on his garments that they "exploded like the fire of two ranks of infantry."

The role of lice in the production of typhus fever was not discovered until a century later. However, Napoleon's surgeons write so much about lice that we wonder if they did not suspect them. They were aware, however, that typhus was highly contagious. One surgeon observed that York's Prussians, who had not been in Moscow at all, had no cases of typhus fever until they followed the retreating French. Another army corps had no typhus until it passed along a road previously used by the French. After a march of fourteen miles on this road, there were fifteen to twenty men in each company ill from this disease.

Napoleon's disaster in Russia was not the first time he had been defeated by disease. His previous campaign in Syria had ended in retreat before an advancing epidemic of bubonic plague. History records that Napoleon mingled freely among the plague-infested soldiers at Jaffa to show his contempt for the disease and that his surgeon, Desgenettes, inoculated himself with pus from the buboe of a sick soldier, to prove that the disease was not contagious. Desgenettes remained perfectly well, probably because, as we now know, the pus from plague boils often contains very few plague bacilli.

The ravages of typhus, typhoid, and dysentery did not cease with the end of Napoleon's Russian campaign. The retreating army scattered pestilence far and wide. Typhus fever broke out in East Prussia and caused the death of 20,000 inhabitants. From East Prussia it spread rapidly to other parts of Germany, to Silesia, Saxony, Bavaria, and the Rhineland. Prinzing estimates that 10 per cent of the population of Germany contracted typhus fever, and 200,000 to 300,000 died. From Germany it spread to France, where it also caused an appalling toll. In Metz all the physicians contracted the disease and many of them died. In the Department of Moselle alone, more than 10,000 died from typhus, this figure not including soldiers.

Typhoid fever, long confused with typhus, has caused a greater number of deaths than typhus in the armies of nations whose hygienic standards do not tolerate the presence of lice.

In the American Civil War, 186,000 troops of the Union Army died of disease as compared with 44,000 killed in battle and 49,000 who died of wounds. Typhoid fever caused the death of 36,000 soldiers.

The health record of the American Army during the Spanish-American War gives us no cause for pride. During this minor struggle sixty-seven officers and 1,872 men died of disease, and twenty-nine officers and 440 privates were killed in action. The death rate in the army camps became so high as to constitute a national scandal. A commission headed by Walter Reed. was appointed to study sanitation in the army camps. They found that "more than ninety per cent of the volunteer regiments developed typhoid fever within eight weeks after they came into camp."

The report of this commission remains a monumental contribution to our knowledge of typhoid fever. They found that the most important carriers of the infection were flies, which feasted on the dejecta of patients and then crawled over the food in the mess halls. Ignorant, untrained hospital orderlies also spread the disease. Without even washing their hands, many of these orderlies left the hospital wards and walked into the mess hall where they prepared food for the soldiers.

The typhoid bacillus was discovered eighteen years before the Spanish-American War, the Widal reaction had been in use for three years, the mode of infection and methods of prevention had long been known. Yet in an army of 107,973 men, there were 20,738 cases of typhoid fever.

The Boer War was an even greater blot on the pages of the history of sanitation. The British had apparently profited little by the American experience. In this war, the British Army lost 22,000 men, of whom 8,000 had been killed in battle or died of wounds, while 14,000 died of preventable diseases. There were 57,684 cases of typhoid fever with 8,022 deaths. The typhoid bacillus had killed more men than the Boers.

The high typhoid fever rate in the British Army during the Boer War, seems inexcusable in the light of present knowledge. Three years before the Boer War, Wright had demonstrated the protective value of antityphoid vaccines, and two years later had inoculated four thousand men of the British Indian Army, with excellent results. The British Army in South Africa authorized—permitted is perhaps a better word—voluntary vaccination. The vaccination was carried out in a rather haphazard fashion, records were poorly kept and often lost. The army authorities finally forbade the inoculations altogether.

Two years after the close of the Boer War a Royal Commission appointed to study the value of antityphoid vaccination issued a favorable report which rehabilitated the procedure of antityphoid inoculation. It came too late to aid the typhoid victims of the Boer War, but it carried a poignant message to the armies in future wars.

With the turn of the century there came a turn in the health record of armies engaged in war. During their war with China in 1894 and 1895, the Japanese lost four times as many soldiers from disease as from bullets. Indeed, this had been the record of armies for decades. These figures appalled the Japanese government, which reorganized its military medical service and

sent thousands of young Japanese all over the world to study the latest advances in sanitation. Japan, to quote Major Seaman, writing in 1905, "even had the temerity (strange as it may seem to an English or American Army official) to grade her medical men as high as officers of the line, who combat the enemy that kills only 20 per cent."

The report of the Japanese Army at the end of the Russo-Japanese War showed the wisdom of their policy. Seven per cent died in battle; 1.5 per cent died of disease.

The first World War proved that the United States Army had profited by its own mistakes in the Spanish-American War and by the mistakes of the British in the Boer War. The lessons of the Russo-Japanese War had also been studied and learned.

Typhoid fever, the great scourge of past wars, claimed only 213 victims in the American Army of 4,000,000. If the typhoid death rate of the Spanish-American War had prevailed, more than 68,000 soldiers would have lost their lives from this disease. Surgeon General Ireland of the United States rightly attributed this decline to vaccination and improved sanitation, including careful examination of cooks and food handlers.

Tetanus caused only four deaths in the American Army, less than two out of every hundred thousand wounded. This figure is especially noteworthy, since the British in the early years of the war had 32 cases in every thousand wounded. Prophylactic injections of tetanus antitoxin in all the wounded explained the astonishing American figures.

Typhus fever did not cause a single death in the American Army. The figures from Russia were quite different and, indeed, appalling. During the war and the succeeding revolution, sanitation broke down completely in Russia. There were more than twenty million cases of typhus in Russia, with three million deaths.

The medical experiences of the World War demonstrated the great advances made in the prevention, control, and cure of certain infectious diseases. Two great groups of diseases remained, however, untamed. "The sputum-borne infections," observed Colonel Garrison, "particularly the pneumonias, remained the insoluble problem of the war." To this must be added streptococcic infections, which caused 70 per cent of the fatal infections in the British Army. The discovery of the therapeutic effect of the sulfonamides in 1933 bids fair to mark the beginning of the solution of what Garrison called the insoluble problem of the World War.

While we think of disease and war, we are prone to think only of infectious diseases and gloss over or neglect the role that disease due to faulty or insufficient nutrition plays. Prominent among these diseases is scurvy.

Scurvy appeared in the armies of the First Crusade. Jacques de Vitry, one of the crusaders, wrote an excellent account of this disease as it appeared at the siege of Damietta in 1219, and notes that it was a "pestilence, against which the doctors could not find any remedy in their art." Later in the Seventh Crusade of 1248, the army of Louis IX of France was attacked by this disease, and no one, reading the account of Sieur de Joinville, can doubt that scurvy was largely responsible for the disaster that befell Louis' army.

In 1535 Jacques Cartier's expedition to Canada suffered greatly from scurvy and probably would have perished to the man, except for the aid of the Indians, who told him that a decoction of the bark and leaves of the fir tree would cure the disease. Cartier's physicians had been powerless in the extremity, so we can understand his oft-quoted remark that the decoction of fir "wrought so well, that if all the physicians of Montpellier and Louvain had been there with all the drugs of Alexandria, they would not have done so much in one year as that tree did in six days."

The work of James Lind, who found that lime juice added to sailors' rations would prevent scurvy, is one of the great discoveries in medicine. Yet, incredible as it seems today, Lind waited forty-two years before he saw his measure put into effect by the British admiralty. The French seemed even more blind than the British to the importance of Lind's discovery. Larrey relates that Napoleon's army in Egypt was decimated by scurvy in 1799, incidentally forty-six years after Lind's discovery. The addition of lime juice to the rations of the French soldiers in Egypt might have proved a decisive factor in the campaign.

Since the demonstration that citrus fruits, added to rations, prevents scurvy, this disease has lost its military importance. In the recent Italo-Ethiopian War, there was not a single case of scurvy in the Italian Army, although the Ethiopian Red Cross reported over thirty thousand cases of scurvy on the Somaliland front alone.

The medical history of the present war remains to be written. Much can happen in the domain of diseases as well as in the field of strategy. Thus far, the glimpses we have had are encouraging.

The plaster of Paris method of treating wounds, as advocated by Orr and given a wide application in the Spanish Civil War, has had a marked effect in lowering the death rate from compound fractures. The sulfonamide drugs have been employed with gratifying effects in streptococcus infections, in pneumonia, and in meningitis. Sulfathiazole has proved a great value in curing as well as in preventing gonorrhea, a disease which in the last war kept several divisions constantly hospitalized.

Typhus fever in the present war has not been a serious menace. An epidemic of typhus has not appeared in Serbia as in the last war, probably because sanitation has improved there and the Germans are remembering the lessons of the previous epidemics. The same also holds for the occupied portions of Russia. The great epidemic in Russia during the last war appeared principally during the chaos of revolution.

While it is apparent today that methods of destroying life are more effective than ever before in history, it is comforting to the physician to know that methods of saving life are more numerous and more effective.

BOOK NOTICES

Books Dealing With War Medicine

War Medicine*
A Symposium

IT IS appropriate in the present emergency to republish collected papers on a subject of such tremendous immediate importance as war medicine, papers which have been scattered in a dozen or more journals, some in this country, some in Canada, and many in England. Most of the authors who have written on subjects dealing with war medicine are now actively engaged in the war. It would be folly to ask them to contribute rehashes of what they have already written, merely for the sake of calling it a new and original contribution. Such a procedure would not contribute to the prosecution of the war. The republication of outstanding contributions has been adapted with some of the articles appearing in the two War Medicine Numbers of THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE in order to supplement and round out the symposium of original contributions.

The volume under review, "War Medicine, A Symposium," edited by Commander Pugh and Doctors Podolsky and Runes, is especially effective in presenting the most recent advances in war surgery. This fills approximately two-thirds of the volume. There are two other sections, "Aviation and Naval Medicine" and "General Medicine." The collected writings round out the entire field, but the volume is recommended especially for those dealing with war surgery.

A Short History of Nautical Medicine†

READERS of Captain Roddis' *A Short History of Nautical Medicine* will not be limited to naval physicians. This thoroughly interesting volume will appeal to army physicians as well as to naval medical men, to nonmedical naval men and seafarers in general, to historians and to nutritionists. Starting with the sparse records of maritime medical service used by the ancients, it traces the contributions in this field down to modern times. Captain Roddis lists some of the most important as (1) the conquest of scurvy (a fascinating story); (2) the distillation of drinking water; (3) the introduction of smallpox vaccination among sailors; (4) improved personal hygiene; (5) the institution of quarantine (and we learn here that the word is derived from the forty days of detention); (6) improvements in ventilation on shipboard; (7) the institution of preliminary physical examinations; (8) improvements in medical service on shipboard; (9) the development of naval hospitals and hospital ships, etc. The author develops the history of nautical medicine during the last few hundred years, especially through the story of the British Navy and the United States Navy. The book abounds in interesting anecdotes, especially in these two groups.

*War Medicine. A Symposium. Editor Winfield Scott Pugh, M.D., Commander, M. C., U. S. Navy, Retired; formerly surgeon, City Hospital, New York; Associate Editor Edward Podolsky, M.D.; Technical Editor Dagobert D. Runes, Ph.D. Cloth, 565 pages, $7.50. F. Hubner & Company, Inc., New York, 1942.

†A Short History of Nautical Medicine. By Louis H. Roddis, M.D., Captain, Medical Corps, United States Navy. Cloth, 12 illustrations. 359 pages, $3.00. Paul B. Hoeber, Inc. Medical Book Department of Harper and Brothers, New York and London, 1941.

Medical Manual of Chemical Warfare*

THE *Medical Manual of Chemical Warfare* is a reprinting of an official British manual. It contains a general description of war gases and detailed discussion of the different types, such as blister gases, choking gases, paralyzing gases and harassing gases. The list is quite complete. The volume describes methods of protection, recognition, and first-aid treatment. With deep regret we are forced to say that this volume will probably be very useful in the next years. The approach is clinical and practical.

What the Citizen Should Know About Wartime Medicine†

THE most recent of a series of twelve volumes to date of *The Citizen Series* is *What the Citizen Should Know About Wartime Medicine.* The books include subjects, such as what the citizen should know about the Army, the Navy, the Coast Guard, the Marines, Air Forces, Civilian Defense, submarine warfare, etc. Colonel Darnall and his associate author, V. I. Cooper, have given us an adequate presentation of the many functions of the medical services in the armed forces. This includes the organization of the medical department, aviation medicine, military psychiatry, sanitation, hygiene, the prevention and control of infectious diseases, the venereal disease problem, and war surgery. So much must be said in these fields that there is no space for history of the development of medical problems in past wars.

The book is written primarily for the layman. The diversified activities of the medical departments sometimes make it difficult for an officer in the medical service assigned to a highly specialized task to realize how many other problems are being prosecuted in his own department of the Army or Navy. For this reason, the volume will make interesting reading for men in the medical services.

Dermatologic Therapy in General Practice‡
Manual of Dermatology

TWO books on dermatology, written in part for the services, should be reviewed together, not so much because Dr. Sulzberger is coauthor of both books but because in actual practice the volumes will be found to be supplementary to each other.

The *Manual of Dermatology* has been issued under the auspices of the Committee on Medicine of the Division of Medical Scienes of the National Research Council and written by Donald M. Pillsbury, Marion B. Sulzberger, and Clarence S. Livingood. Prefatory notes by the Surgeon Generals of the Army and Navy explain the need for such a volume for use in the armed services. Nearly 10 per cent of all admissions to sick list and 10 per cent of all days lost on account of sickness are due to dermatologic maladies. This volume is in essence a vade mecum for physicians who are called upon to treat skin diseases. Most of the information and directions are tabulated and are very brief. The illustrations are so numerous and so excellent as to make it almost a dermatologic atlas. Important directions and cautionary remarks are emphasized by being boxed in.

The other volume *Dermatologic Therapy in General Practice* by Lieutenant Commander Marion B. Sulzberger and Dr. Jack Wolf is somewhat larger but is still of conveniently small size and presents adequately minute details of the methods of applying treatment

*Medical Manual of Chemical Warfare. Reprinted by permission of the Controller of His Britannic Majesty's Stationery Office, revised edition, 1942. Cloth, 121 pages, $2.50. Chemical Publishing Company, Inc., Brooklyn, N. Y.

†What the Citizen Should Know About Wartime Medicine. By Joseph R. Darnall, M.D., Lieut. Col., M. C., U. S. Army; and V. I. Cooper. Illustrations by André Fandot. Cloth, 227 pages, $2.50. W. W. Norton & Company, Inc., New York, 1942.

‡Dermatologic Therapy in General Practice. By Marion B. Sulzberger, M.D., Lieut. Comdr., M. C., U. S. N. R., Assistant Clinical Professor of Dermatology and Syphilology, Skin and Cancer Unit of the New York Post-Graduate Medical School and Hospital of Columbia University; Associ[illegible] iogist, Montefiore Hospital, New York City; and Jack Wolf, M.D., [illegible] and Syphilologist, Skin and Cancer Unit of the New York Post-Gr[illegible] and Hospital of Columbia University; Director of Dermatology, New York City Cancer Institute. Cloth, 632 pages. The Year Book Publishers, Inc., Chicago, 1942.

Manual of Dermatology. Issued under the Auspices of the Committee on Medicine of the Division of Medical Sciences of the National Research Council. By Donald M. Pillsbury, M.D., Marion B. Sulzberger, M.D., Clarence S. Livingood, M.D. Cloth, 421 pages, $2.00. W. B. Saunders Company, Philadelphia & London, 1942.

which are briefly touched upon in the *Manual of Dermatology.* The approach to dermatologic therapy in this book is definitely different from that of most textbooks on skin diseases. While the importance of accurate diagnosis is recognized and diagnostic discussion appears in the second portion of the book, emphasis in the first part is placed upon selection of the appropriate treatment based upon the character of the skin manifestation rather than upon its nosologic classification. In other words, one can apply the appropriate type of treatment in the great majority of cases even though one may not have had sufficient dermatologic experience to make the correct diagnosis in some unusual dermatosis.

These two volumes will be of greatest value when used together.

Fundamentals of Psychiatry*

WHEN millions of men are drafted into the armed forces, large numbers of neurotics, psychoneurotics, and psychotics are bound to be overlooked in the preliminary draft appraisal and admitted into the Army, Navy, or Marines. The tremendous stress of service life even without the emotional problems of combat activity serves to bring out psychotic tendencies in many who might have gone years longer before becoming ill. Military psychiatry is a vast field in its own right. Dr. Strecker's small volume, *Fundamentals of Psychiatry*, is, as its name implies, a synopsis of the subject of psychiatry and methods of psychiatric examination and treatment, in which he has incorporated much discussion of psychiatric problems of wartime.

War Gases†
Their Identification and Decontamination

THE British book, *Medical Manual of Chemical Warfare*, reviewed elsewhere, presents a comprehensive discussion of the uses and damaging effect of war gases. The present volume by Morris B. Jacobs, formerly Lieutenant in the U. S. Chemical Warfare Service Reserves and Chemist in the Department of Health of The City of New York, presents a similar discussion which the reviewer feels is better classified. The volume deals first with the classification of the chemical agents, then with the physical characteristics and physiological responses to war gases, and next with the effect of war gases on inanimate material, such as food and water. There is a section on sampling of gases and methods for analytic determination of their nature. The final section deals with methods of decontamination. Before the war is over, this volume will probably be widely used as a quick reference manual.

Civilian Health in Wartime‡

DR. DIEUAIDE'S volume, *Civilian Health in Wartime*, is in great measure a treatise on preventive medicine applied to the individual and to groups, which would be equally readable in peacetime. Written primarily for the layman, it provides at the same time an excellent refresher or orientation manual for the physician, nutritionist, and public health worker. The author discusses the effect of war on civilian health. He gives considerable attention to nutrition and diet. He next discusses infectious diseases and methods of control.

The importance of proper clothing and housing conditions is discussed. Maternal and child welfare, as well as geriatric problems, are presented in an easily readable style which should be of real interest to the intelligent layman. Other subjects include recreation, occupation, morale, and a chapter about doctors and nurses, how they work and what they can be expected to accomplish.

---

*Fundamentals of Psychiatry. By [illegible]r, M.D., Sc.D., F.A.C.P., Professor sity of Pennsylvania; Psychiatrist to [illegible] Hospital; Attending Psychiatrist, Psychopathic Division, Philadelphia Gener[illegible] 15 illustrations, 201 pages, $3.00. J. B. Lippincott Company, Philadelphia, [illegible] 1942. of Psychiatry and Chairman of the Department, Undergraduate School of Medicine, University of Pennsylvania

†*War Gases. Their Identification and Decontamination.* By Morris B. Jacobs, Ph.D., Food, Drug and Insecticide Administration, U. S. Department of Agriculture, 1927, Chemist, Department of Health, City of New York, 1928; formerly Lieut. U. S. Chemical Warfare Service Reserve. Cloth, 180 pages, $3.00. Interscience Publishers, Inc., New York, June 26, 1942.

‡Civilian Health in Wartime. By Francis R. Dieuaide, M.D., Associate Professor of Medicine, Harvard Medical School, Massachusetts General Hospital. Cloth, 328 pages, $2.50. Harvard University Press, Cambridge, Mass., 1942.

The Journal of Laboratory and Clinical Medicine

Vol. 28 | March, 1943 | No. 6

CLINICAL AND EXPERIMENTAL

LESIONS IN THE TISSUES OF THE BODY FOLLOWING SULFONAMIDE THERAPY*

Herbert J. Schattenberg,† M.S., M.D., F.A.C.P., and William H. Harris, Jr., B.A., M.D., New Orleans, La.

Not since the introduction of arsphenamine and neoarsphenamine many years ago, has a therapeutic agent received such a welcome and cordial reception as when the sulfonamides were introduced to the profession. Originally introduced to combat serious and grave infections caused by the streptococci, especially *Streptococcus hemolyticus,* the value of these drugs was soon established in pneumonia, meningitis, various bacteriemias, genitourinary tract infections, bacillary dysentery and any number of other disease entities. There is hardly a single specialty in the medical profession which does not make use of one or more of the sulfonamide drugs. Unfortunately, the laity has become too well acquainted with these drugs, looking upon them as a cure for any and all ailments, and are therefore using them haphazardly and indiscriminately. Much harm has resulted in many instances from such usage.

As a matter of historical interest, one of the sulfonamides, sulfanilamide (para-amino-benzene-sulfonamide) has been known for many years. Gelmo,[1] working on the chemistry of azo dyes, made it synthetically in 1908. Eisenberg,[2] in 1913 called attention to the bactericidal properties of certain azo dyes. Among the various azo dyes tested were pyridium, chrysoidine, and scarlet red. In 1935 chemists working under Domagk[3,4] produced prontosil I and prontosil II. Prontosil itself possesses poor bactericidal properties, but when its molecule is broken down in the body, a potent product, sulfanilamide, is set free. Trefouel,[5] and Fourneau[6] in France confirmed the contention that the azo dye upon being broken down in the body liberates sulfanilamide. Interest in these

*From the Department of Pathology, Tulane University School of Medicine and the Charity Hospital of Louisiana at New Orleans.

Received for publication, July 20, 1942.

†Now at the Medical and Surgical Memorial Hospital, San Antonio, Texas.

new drugs first developed in America in 1936. The work in this country by Mellon,[7] and Long and Bliss[8] is well known.

Nothing is definitely known about the therapeutic action of the sulfonamides. Theoretically, it may be assumed that the drug acts by: 1. A neutralization of bacterial toxins. 2. It stimulates the cellular and humoral defensive mechanism of the host against the infectious bacteria. 3. It acts as a germicide and destroys the invading and circulating bacteria. This is unlikely since it has been shown by Selbie[9] that simultaneous administration of para-amino-benzoic acid and sulfanilamide in experimental animals does not prevent a fatal outcome, the action of the latter being antagonized by the former. One molecule of para-amino-benzoic acid inhibits the action of 23,000 molecules of sulfanilamide. Janeway[10] has shown that the addition of small amounts of para-amino-benzoic acid to blood cultures inhibits the action of sulfanilamide and thereby permits the bacteria present to grow and flourish. This would seem then that sulfanilamide acts merely as a bacteriostatic agent, giving other defensive forces of the body an opportunity of assisting them in ridding the body of infection.

Many articles have appeared in the medical literature extolling the virtues of the various members of the sulfonamide group of drugs in the treatment of different infectious diseases. Practically every medical program offers one or more scientific papers describing new uses or discussing clinical results obtained with these drugs. As so often happens with new therapeutic agents which appear to accomplish unusually favorable results, no thought is given at first to the fact that in some instances they may also do considerable harm. This has also been true of the arsphenamines, cinchophen, atabrin, and many other drugs when they were first introduced. It is true that all new drugs are at first thoroughly controlled for toxicity and other properties. These tests are carried out, in the majority of instances, on normal experimental animals. Let us not lose sight of the fact that an experimental animal often reacts differently to a drug than does a human being. Of more importance is the fact that in the instance of the patient extensive injury may already have occurred in the important parenchymatous organs as a result of serious infectious disease. Naturally a drug which itself may be toxic adds insult to injury in such an instance.

Recently numerous reports have appeared in the medical literature describing unfavorable reactions and even death as a result of sulfonamide therapy. Various types of skin lesions, nephritis, focal necrosis, and acute yellow atrophy of the liver, involvement of the central nervous system, myocarditis, anemia, leucopenia, and various other conditions have been described.

Lesions of the skin appear as one of the most frequent complications and may be a manifestation of an allergic or toxic manifestation or otherwise as a drug idiosyncrasy. Wien and Lieberthal[11] describe a pemphigus, foliaceous-like eruption following the use of sulfanilamide and sulfapyridine. Weinstein and Domm[12] report a case of a patient with pneumonia receiving a total of 15 Gm. of sulfathiazole. The patient developed a severe, acute exfoliative dermatitis. "Other than an overwhelming toxemia resulting from the widespread and severe involvement of the skin, no satisfactory cause of death was found." Volini, Levitt, and O'Neil[13] present a case report and discuss cutaneous and conjunctival manifestations of sulfathiazole intoxication. The severity of skin

lesions with extensive necrosis is well shown and discussed in a case report by Klingensmith.[14]

Lesions of the liver in sulfonamide poisoning have not received the attention they deserve. This is probably due to the fact that in the majority of instances such a diagnosis is arrived at only at autopsy. It has been our experience that

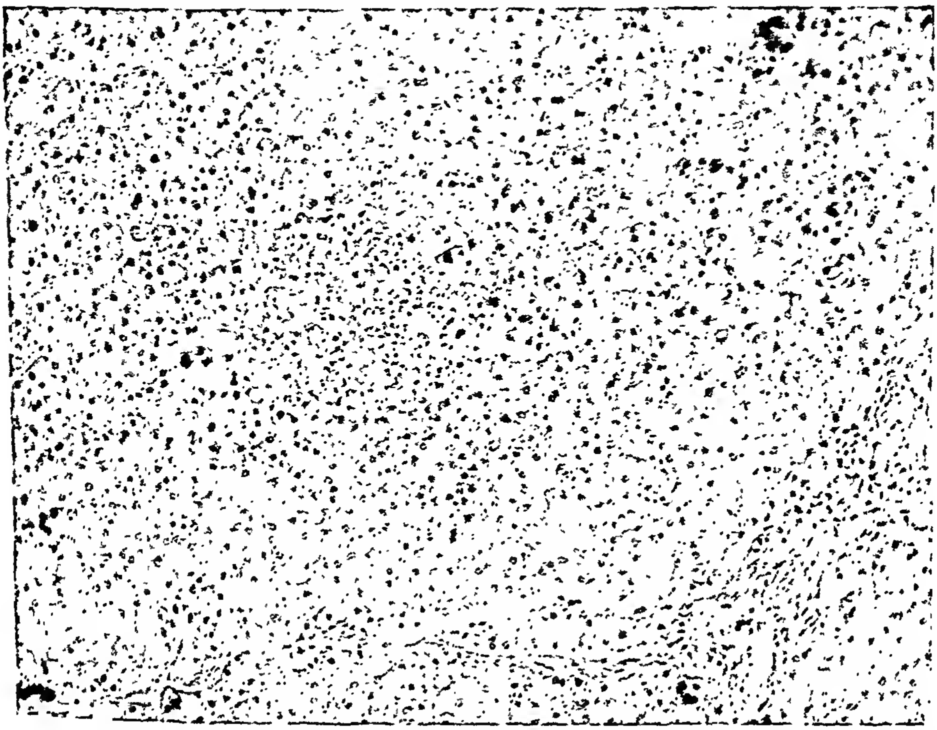


Fig. 1.—Photomicrograph of section of human liver. Note central area of focal necrosis. In other areas isolated liver cells likewise show necrosis. (×400.)

Fig. 2.—Photomicrograph of section of human liver showing extensive acute atrophy. Only a few isolated liver cells remain, and from the histology present it is difficult to tell that this is liver tissue. (×400.)

lesions in the liver are quite common and are frequently found at autopsy. The changes which we have noted at autopsy are severe cloudy swelling of liver cells, fatty degeneration, focal necrosis (Fig. 1), and in one instance there was encountered a case of acute yellow atrophy (Fig. 2).

Fig. 3.—Section of kidney showing tubules filled with blood cells and albumin casts. (×500.)

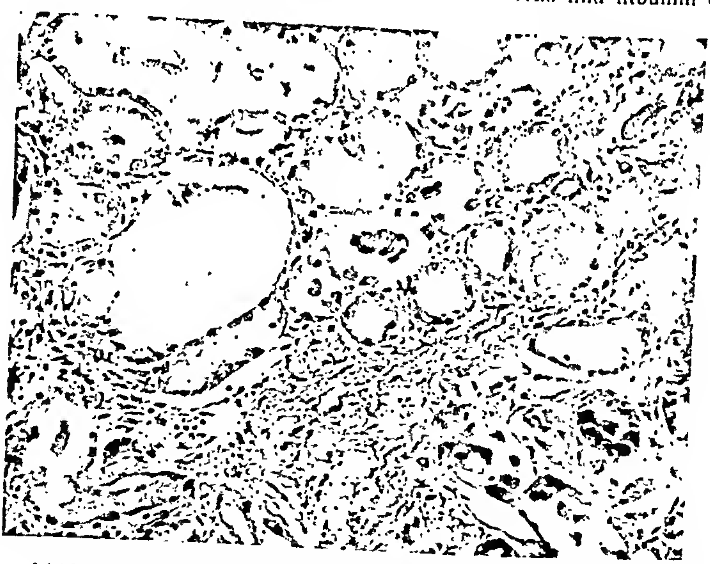


Fig. 4.—Section of kidney showing marked distention and dilatation of some of the tubules due to blockage by sulfapyridine crystals. (×500.)

The literature contains numerous reports of liver damage following sulfonamide therapy. Garvin[15] reports five cases of toxic hepatitis of patients who had primarily received sulfanilamide. A case of acute toxic necrosis of the liver following the use of sulfanilamide is described by Russell.[16] Watson,[17] in an excellent study, discusses the alteration of hemoglobin metabolism and hepatic function in a large series of cases receiving sulfanilamide and sulfapyridine.

Effects of these drugs upon the central nervous system are infrequently reported. One of the most recent reports is that of Little,[18] who describes the nervous and mental effects of the sulfonamides. Bieter and co-workers[19] carried out a series of experiments on chickens. Extensive peripheral nerve injury was noted. These investigators also present a case report wherein a patient developed deafness and loss of function of the right lower extremity following the use of sulfathiazole. These symptoms disappeared and function was restored after the drug was discontinued.

Fig. 5.—Gross picture of monkey's kidneys, ureters, and urinary bladder. Note marked ureteral dilatation and hydronephrosis on right due to blockage by sulfapyridine deposits. Ureter on left had been opened longitudinally and shows normal diameter of this structure.

Kidney injury is one of the most common complications encountered in patients receiving sulfonamide therapy. Instances of anuria, hematuria, nitrogen retention, and renal colic have been reported. Tubular casts we have noted are not an uncommon finding (Fig. 3). The insoluble acetyl derivative of these drugs forms crystalline deposits which may block the tubules (Fig. 4) or be so extensive as to cause ureteral blockage with hydronephrosis. It has been shown by Schwartz and co-workers[20] that crystalline precipitation is more likely to occur in acid urines. In a series of patients 28 per cent of those with alkaline urines showed crystalluria, while 70 per cent of those with an acid urine showed a similar precipitation. Alkalinization, therefore, seems an essential procedure to prevent precipitation and urinary tract blockage. The appearance of the

urinary acetyl derivatives of the various sulfonamides is well shown in the discussion by Lehr and Antopol.[21] It is usually not difficult to demonstrate crystalline depositions in tubular structures in frozen section preparations of kidney tissue. Passage of kidney sections through the various steps of the paraffin technique followed by hematoxylin-eosin staining dissolves out the sulfonamide crystals. Museum specimens of kidneys, ureters, and urinary bladders soon become worthless due to the fact that crystalline deposits of these drugs slowly dissolve in the Kaiserling solutions.

It was our privilege recently to examine the kidneys, ureters, and urinary bladder of a monkey which had died from an infectious disease. In treating the infection the animal had received large doses of sulfapyridine. At autopsy a large amount of crystalline material was found in the bladder and both ureters were completely blocked (Fig. 5) by crystal accumulations resulting in extensive bilateral hydronephrosis, undoubtedly hastening the animal's death. Excellent discussions of urinary tract changes are found in the articles by Winsor and Burch[22] and Antopol, Lehr, Churg, and Sprinz.[23]

Grass, Cooper, and Morningstar[24] have produced the experimental lesions of glomerulonephritis in rats with sulfapyridine. The lesions reported consisted of swelling and proliferation of the capsular epithelium, basement membrane thickening, hyaline degeneration of the tufts, endothelial proliferation of the tuft vessels, and adhesions of the tufts to Bowman's capsule.

Grave disturbances in the hematopoietic system may be encountered in sulfonamide therapy. This may take the form of a rapidly fatal acute hemolytic anemia, a fatal agranulocytosis, or less serious manifestations of these diseases. In the instances of agranulocytic angina, it is likely that these drugs may act in a manner similar to certain barbiturates, such as amytal, barbital, dial, or neonal. These are all "coal tar" derivative or "benzene ring" drugs, similar to the sulfonamides, and have a markedly depressing effect in some patients upon the leucoblastic centers in the bone marrow. It is needless to say that frequent leucocyte and erythrocyte counts and hemoglobin determinations during the course of therapy will forestall in many cases these serious complications. Spring and Bernstein,[25] Jennings and Southwell-Sander,[26] and many others report important findings from the hematologic standpoint in these cases.

An important lesion which is too infrequently mentioned is interstitial myocarditis. A review of the literature reveals the fact that attention is focused solely upon lesions of the kidneys, skin, liver, and hematopoietic system. It is very likely that changes in the myocardium have been overlooked in human autopsy material and also in experimental animals. It is hoped that the few articles appearing on interstitial myocarditis in patients receiving the sulfonamide drugs will stimulate further interest in this much neglected topic.

Our interest in interstitial myocarditis was occasioned by a perusal of the experimental work and autopsy study of human hearts by French and Weller.[27] We have noted histopathologic findings in human hearts similar to those reported by these authors. These consisted of eosinophilic cellular infiltrations between the muscle fibrils (Fig. 6), and in some cases forming collars about the small branches of the coronary vessels (Fig. 7). The cells were mainly large

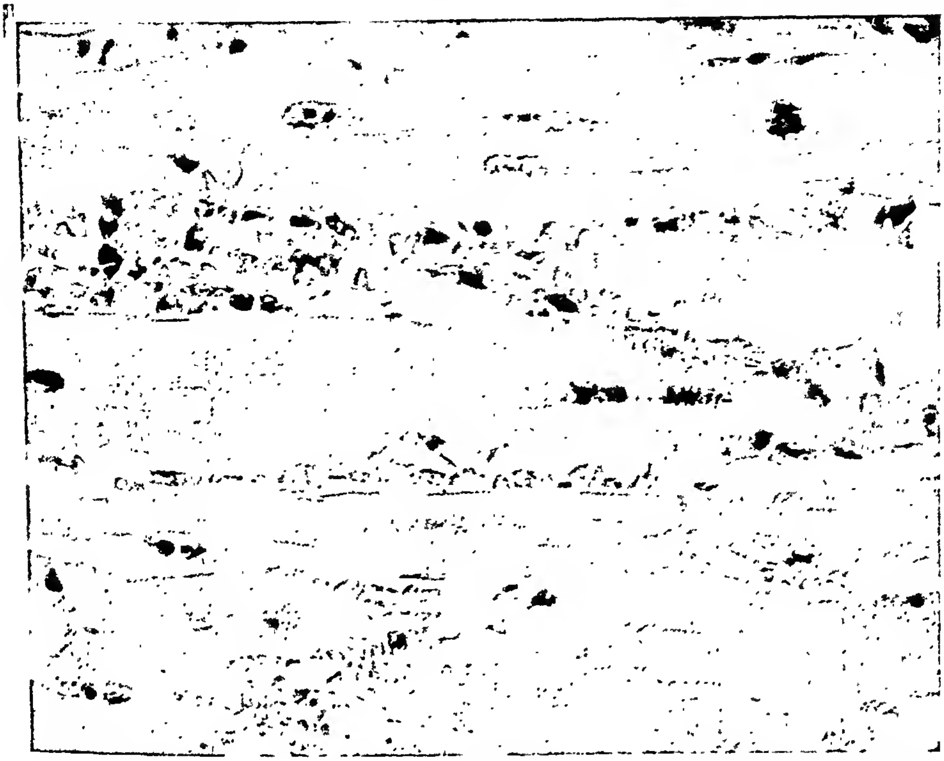


Fig. 6.—Photomicrograph of section of human heart showing interstitial accumulations of blood cells. Most of these cells are large mononuclears with pink staining cytoplasm. There are present also some neutrophiles and a few erythrocytes. (×550.)

Fig. 7.—Photomicrograph of heart section showing perivascular infiltration with cells of a type similar to those described in Fig. 6 (×400.)

mononuclear cells with pink-staining cytoplasm and a considerable number of polymorphonuclear leucocytes, many with an eosinophilic cytoplasm.

In the study by French and Weller 126 cases, or 44.5 per cent, of the 283 receiving sulfonamide drugs showed a significant interstitial myocarditis. While the series studied by us so far is small, it probably will not reveal as high a percentage of interstitial myocarditis as reported by French and Weller.[27] In not any of our cases could the characteristic eosinophilic infiltrations be attributed to any other agent or cause than the sulfonamides.

Electrocardiographic changes were noted in a series of patients receiving these drugs, as reported by Scheinberg, and Ingle,[28] and Dazzi.[29]

In passing it might be appropriate to mention a word about the large number of deaths which followed the use of elixir of sulfanilamide in September and October of 1937. This whole matter is excellently reviewed in a Special Article[30] in the *Journal of the American Medical Association*. It should be re-emphasized that sulfanilamide was not to blame for these deaths, but its vehicle, diethylene glycol, a highly poisonous substance was responsible.

The tremendous popularity of the sulfonamides is shown by referring to the statistics of Drug Trade News, as quoted by Pruitt.[31] The manufacturers' sales in the United States in 1940 of sulfanilamide alone amounted to 543,802 pounds. What percentage of this was disposed of on physicians' prescriptions is difficult to say. The danger of the indiscriminate use of these drugs was pointed out in an Editorial[32] by the *Journal of the American Medical Association* as far back as 1937: "The startling news reports that the administration of sulfanilamide will 'cure' gonorrhea in forty-eight hours has led to some unpleasant results. Responsibility lies considerably with pharmacists who are willing to sell dangerous drugs to anybody over the counter. In one large city, hospitals have admitted young men with severe sulfhemoglobinemia resulting from self-medication with sulfanilamide. The physician must bear in mind the potential hazards of this drug."

CONCLUSIONS

A study of a large series of cases and a careful review of the literature reveals the important finding that any organ or tissue in the body may be affected through the toxic action of the sulfonamides. In what percentage of cases this occurs is impossible to say. Considering the popularity of these drugs and the tremendous quantity used, amounting to over one-half million pounds of sulfanilamide for 1940 alone, it is not surprising to note some bad effects and even deaths from time to time.

The immediate effects of these drugs upon the skin, liver, kidneys, and nervous system is well understood. It is not possible at this time to say what residual effects may be noted in the myocardium, liver, or other organs years later. Might such changes lead to chronic myocarditis or cirrhosis of the liver? Might they lead to bizarre blood dyscrasia? No one as yet knows the answer.

Greater precaution in the use of these drugs is advisable. Dispensing on physicians' prescriptions only would lead to proper control and discourage abuse and indiscriminate use by the laity. It is certainly not the intent to discourage a drug which has proved its merit. At times, as in grave pneumonias, meningitis, puerperal infections, and otherwise serious bacteriemias it may truly be a "life-

saver." In cases of less gravity or in disease entities in which other therapy may be employed its usefulness is doubtful.

When sulfonamide therapy is indicated and necessary, certain precautions should be observed which may prevent disastrous effects from the toxicity of the drug. The amount of drug and duration of administration should be kept at a minimum consistent with beneficial results. Frequent checking to determine proper concentration of the drug in the blood is essential. Frequent erythrocyte and leucocyte counts and hemoglobin determinations with an immediate discontinuance of medication when there is evidence of important changes in these elements. An evaluation of renal function is necessary because a damaged kidney handles these drugs poorly. Progressive oliguria and hematuria may make continued administration of the drug hazardous. A continued high temperature after other signs of infection have disappeared may be entirely due to the drug. In a number of such cases observed the temperature has dropped to normal following the discontinuance of the sulfonamides. Alkalinization may prevent renal calculus formation. Electrocardiographic tracings may shed light on early myocardial changes following the use of these drugs.

An agent which is toxic for bacteria may in many instances be equally as toxic for the cells of the host and thereby increase the burden rather than lessen it. The normal tissues of the control animal, employed by the pharmaceutical firms, are affected to a lesser extent than the tissues and organs of a sick patient already the seat of cloudy swelling, fatty degeneration, etc., the result of bacterial toxemia.

Undoubtedly, with a check on the injudicious use of the sulfonamides, their discontinuance in diseases wherein the mortality is nil and where other forms of therapy are effective and close observation of the patient in whom their use is thought to be of decided benefit will result in fewer serious reactions and possible death.

REFERENCES

1. Gelmo, P.: Sulphamides of p-Aminobenzenesulphonic Acid, J. Prakt. Chem. 77: 369, 1908. C.A. 2: 2551.
2. Eisenberg, P.: Untersuchungen über halzspezifische Desinfektions-vorgänge. I. Mitt. Ueber die Wirkung von Farbstoffen auf Bakterien. Vitalfarbung-Entwicklungschemmung, Zentralbl. f. Bakt. I. 71: 421, 1913; Abstr. Jahresb. ü. d. Fortschr. d. Tier-chem. 43: 1078.
3. Domagk, G.: Chemotherapie der bakteriellen Infektionen, Angew. Chem. 48: 657. 1935; C.A. 30: 766.
4. Domagk, G.: Ein Beitrag zur chemotherapie der Bakteriellen infectionen, Deutsche med. Wchnschr. 61: 250, 1935.
5. Trefouel, J., Trefouel, J. (Mme), Nitti, F., and Bovet, D.: Activite du p-aminophenylsulfamide sur les infections streptococciques experimentales de la souris et du lapin, Compt. rend. Soc. de biol. 120: 756, 1935.
6. Fourneau, E., Trefouel, J. (Mme), Nitti, F., and Bovet, D.: Chimio-therapie des infections streptococciques par les derives du p-aminophenylsulfamide, Compt. rend. Soc. de biol. 122: 258, 1936.
7. Mellon, R. R., Locke, A. P., and Shinn, L. E.: The Anti-enzymatic Nature of Sulfanilamide's Bacteriostatic Action, Am. J. M. Sc. 199: 749, 1940.
8. Long, P. H., and Bliss, E. A.: The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds, New York, 1939, The Macmillan Co.
9. Selbie, F. R.: The Inhibition of the Action of Sulfanilamide in Mice by para-Aminobenzoic acid, Brit. J. Exper. Path. 21: 90, 1940.
10. Janeway, C. A.: Method for Obtaining Rapid Bacterial Growth in Cultures From Patients Under Treatment With Sulfonamides, J. A. M. A. 116: 941, 1941.
11. Wien, M. S., and Lieberthal, E. P.: Pemphigus Foliaceus-like Eruption Following Use of Sulfanilamide and Sulfapyridine, J. A. M. A. 117: 850, 1941.
12. Weinstein, Mary, and Domm, A. H.: Development of Acute Exfoliative Dermatitis During Administration of Sulfathiazole, J. A. M. A. 117: 607, 1941.

13. Volini, I. F., Levitt, R. O., and O'Neil, H. B.: Cutaneous and Conjunctival Manifestations of Sulfathiazole Intoxication, J. A. M. A. 116: 938, 1941.
14. Klingensmith, W. R.: Necrosis of Skin Due to Sulfathiazole, Texas State J. Med. 37: 313, 1941.
15. Garvin, C. F.: Toxic Hepatitis Due to Sulfanilamide, J. A. M. A. 111: 2283, 1938.
16. Russell, H. K.: Acute Toxic Necrosis of the Liver Following the Use of Sulfanilamide, Ann. Int. Med. 14: 168, 1940.
17. Watson, C. J., and Spink, W. W.: Effects of Sulfanilamide and Sulfapyridine on Hemoglobin Metabolism and Hepatic Function, Arch. Int. Med. 65: 825, 1940.
18. Little, S. C.: Nervous and Mental Effects of the Sulfonamides, J. A. M. A. 119: 467, 1942.
19. Bieter, R. N., et al.: Nervous Injury Produced by Sulfanilamide, J. A. M. A. 116: 2231, 1941.
20. Schwartz, L. F., Flippin, H. F., Reinhold, J. G., and Domm, A. H.: The Effects of Alkali on Crystallauria From Sulfathiazole and Sulfodiazene, J. A. M. A. 117: 514, 1941.
21. Lehr, D., and Antopol, W.: Specific Morphology of Crystals Appearing in the Urine During Administration of Sulfanilamide Derivatives, Am. J. Clin. Path. 12: 200, 1942.
22. Winsor, T., and Burch, G. E.: Renal Complications Following Sulfathiazole Therapy, J. A. M. A. 118: 1346, 1942.
23. Antopol, W., Lehr, D., Churg, J., and Sprinz, H.: Changes in the Urinary Tract and Other Organs After Administration of Three Sulfanilamide Derivatives, Arch. Path. 31: 592, 1941.
24. Gross, P., Cooper, F. B., and Morningstar, W. A.: Glomerulonephritis in Partially Nephrectomized Rats—Relation to Administration of Sulfapyridine, Am. J. Path. 18: 101, 1942.
25. Spring, M., and Bernstein, I.: The Coexistence of Toxic Hepatitis, Acute Hemolytic Anemia, and Renal Damage Following Sulfanilamide Therapy: Report of Two Cases, Ann. Int. Med. 14: 153, 1940.
26. Jennings, G. H., and Southwell-Sander, G.: Anaemia and Agranulocytosis During Sulphanilamide Therapy, Lancet 2: 898, 1937.
27. French, A. J., and Weller, C. V.: Interstitial Myocarditis Following the Clinical and Experimental Use of Sulfonamide Drugs, Am. J. Path. 18: 109, 1942.
28. Scheinberg, D., and Ingle, C. W.: Possible Myocardosis Due to Sulfanilamide, Memphis M. J. 14: 87, 1939.
29. Dozzi, D. L.: Transient Nodal Rhythm Following Use of Sulphanilamide, Am. J. M. Sc. 195: 771, 1938.
30. Special Article, Deaths Due to Elixir of Sulfanilamide—Massingill, J. A. M. A. 109: 1985, 1937.
31. Pruitt, M. C.: The Sulfonamides in Proctology, South. M. J. 35: 598, 1942.
32. Editorial, Sulfanilamide—A Warning, J. A. M. A. 109: 1128, 1937.

INFESTATION WITH STRONGYLOIDES STERCORALIS*

M. B. Levin, M.D., Baltimore, Md.

Human infestation with *Strongyloides stercoralis* in the Middle Atlantic states has been distinctly uncommon.[1] Infested persons in this section of the United States were usually carriers in whom original infestation was traceable to a sojourn in subtropical or tropical regions. Since infestation usually occurred by cutaneous or oral contact with stool from a carrier, it is reasonable to believe that cases occurred in persons who journeyed out of northern climates.

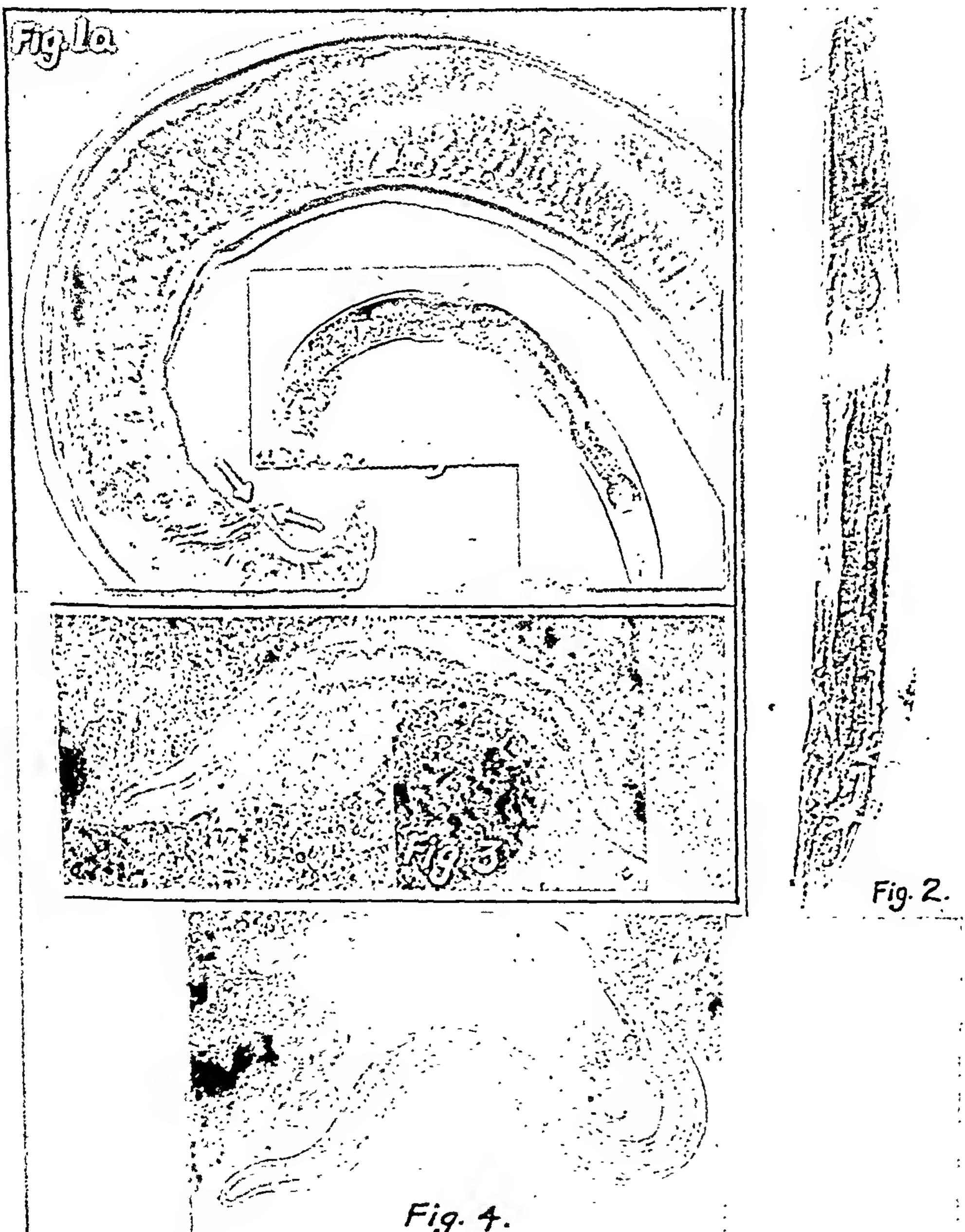
We were fortunate in having secured stool containing *Strongyloides stercoralis* larvae from a patient who had always lived in Maryland, except for a short visit to Virginia about twenty years ago. This case was of interest because the patient's infestation undoubtedly occurred in a nontropical region,

*The Burton-Levin Foundation, Inc.
Received for publication, July 21, 1942.

presumably from infested material from a carrier; and because of the opportunity presented of photographing the rarely observed and only recently discovered male form of the parasite.[2]

ABSTRACT OF REPORTED CASE

The patient presented no definite symptoms of *Strongyloides stercoralis* infestation and only a few very vague gastrointestinal complaints, such as flatu-



Figs. 1-4.—*Strongyloides stercoralis*, adult male and male and female larval forms.

lence, pyrosis, and constipation. He was a 51-year-old policeman who showed during the past year some anginal symptoms that were relieved by belching and morphine; his blood picture revealed a 6 per cent eosinophilia and a mild secondary anemia; and his electrocardiographic findings indicated some myocardial damage. Examination of the stool showed numerous *Strongyloides stercoralis* larvae which was the chief point of interest in this case.

DISCUSSION

In Fig. 1*a*, ×430 magnification, the male sexual organ of the adult form of *Strongyloides stercoralis was plainly seen.* The copulatory spicules and gubernaculum were indicated. In Fig. 1*b*, ×100 magnification, the fertilizing element of the male adult form was indicated at the exit of the sexual organ. Fig. 2, ×430 magnification, showed the male larva with the immature sexual organ indicated. In Fig. 3 and Fig. 4, ×430 magnification, the female larval forms of the parasite were indicated.

SUMMARY

A case is reported in which (1) infestation of *Strongyloides stercoralis* parasites was contracted in a relatively northern region; and (2) the rarely observed male larval and adult form of the *Strongyloides stercoralis* was found in the stool.

REFERENCES

1. Faust, E. C.: Human Helminthology, ed. 2, Philadelphia, 1939, Lea & Febiger.
2. Kreis, H. A.: Studies on the Genus *Strongyloides* (Nematodes), Am. J. Hyg. 16: 450, 1932.

211 West Monument Street

THE EFFECT OF CINCHOPHEN ON BILE FORMATION*

A. L. Berman, E. F. Snapp, A. J. Atkinson, and A. C. Ivy
Chicago, Ill.

On the basis of the results of duodenal drainage experiments it has been reported that cinchophen is a choleretic in man.[1] In the anesthetized dog the drug has a very definite choleretic action[2]; it has no choleretic action in the rabbit.[3, 4] The effect of a single dose or repeated doses in the chronic biliary fistula preparation has not been studied. It was considered important to perform such an experiment because the drug, at least in large doses, is considered to be an hepatotoxic agent.[5]

METHODS

Five healthy biliary fistula dogs which had been carefully observed under dietary control for from two to three months were used. The bile was collected

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.
Acknowledgment is made to the E. L. Dawess and Marjorie Newman Funds for financial aid in this study.
Received for publication, August 3, 1942.

by the Rous-McMaster technique, except as otherwise stated. Three types of experiments were performed: (A) The animals were placed on a control period for from three to five days, the standard diet being fed twice daily without the return of bile or the administration of bile salts or drug. The volume output, and the cholic acid, cholesterol, and total pigment outputs were determined daily. Then the animals were given 1 Gm. of cinchophen in 0.5 Gm. doses with the meal for three days. The cinchophen used was the "pure," or snow white cinchophen prepared by the Calco Company, and was completely soluble in alkaline aqueous solution and devoid of odor; it is less toxic and a different product than that yellowish white product marketed several years ago.[12] The determinations made during the control period were repeated during the test period. However, cinchophen excretion in the bile every twenty-four hours was determined by the method of Bradley.[2] The diet consisted of a commercial dog food (Pard) which was supplemented with dried yeast and cod-liver oil, the dogs consuming the same amount of the diet daily. (B) The second type of experiment consisted in giving cinchophen as above, except it was given for a period of fourteen days. (C) The third type of experiment consisted of giving cinchophen and then returning all bile formed every hour. The detailed method will be given later.

TABLE I

THE EFFECT OF 1 GM. OF CINCHOPHEN DAILY FOR THREE DAYS ON CHRONIC BILIARY FISTULA DOGS, NO BILE OR BILE SALTS BEING GIVEN

DOG NO.	WT. (KG.)	VOLUME (C.C./24 HR.)			CHOLIC ACID (MG./24 HR.)			PIGMENT (MG./24 HR.)			CHOLESTEROL (MG./24 HR.)			RECOVERY OF CINCHOPHEN	
		C	T	% CHG.	C	T	% CHG.	C	T	% CHG.	C	T	% CHG.	TOTAL OUTPUT (GM.)	% REC.
2	10.0	131	266	+103	901	726	-19	88	77	-13	12	16	+ 33		
		137	260	+ 90	1,241	1,268	+ 2	123	124	0	12	18	- 50	2.25	75
3	11.0	130	282	+117	1,472	1,136	- 9	150	120	-20	13	22	+ 69		
		143	287	+100	1,518	1,516	0	133	140	+ 5	15	23	+ 53	2.11	70
4	8.4	122	287	+135	1,346	1,441	+ 7	109	133	+22	12	18	+ 50		
		123	280	+127	1,337	1,561	+16	101	151	+50	13	22	+ 69	1.88	63
5	11.1	140	248	+ 77	1,139	1,175	+ 3	103	126	+22	16	35	+118		
		132	282	+113	1,218	1,257	+ 3	100	138	+38	16	34	+112	1.84	61
6	12.0	130	263	+102	1,420	1,226	-13	85	138	+62	12	25	+108		
		130	277	+113	1,421	1,422	0	120	160	+33	18	43	+138	1.98	66
Average 5 dogs —10 tests		132	273	+106	1,301	1,030	0	111	131	+18	14	26	+ 85		
3 Gm. ketochol		126	251	+ 99	1,468	1,568	+ 6	114	107	- 7	11	14	+ 27	0.728	24
3 Gm. decholin		128	264	+106	1,473	1,647	-12	114	129	+13	11	8	- 27	0.835	28

RESULTS

A. The Effect of Cinchophen Administration for Three Days.—On the average, 1 Gm. of cinchophen daily, given as two doses of 0.5 Gm. with the morning and evening meal, increased the volume output of bile over the control by 106 per cent (Table I). The range of increase was from +77 to +136 per cent above the control output. The bile was clear and odorless, and at no time resembled the bile obtained in the presence of an infectious hepatitis. The choleresis pro-

duced was analogous to that obtained with 3 Gm. of dehydrocholanic acid salts (ketochol) or of sodium dehydrocholate (decholin, sodium).[7]

On the average *cholic acid synthesis* was not changed, although the individual tests show a range of from −19 to +16 per cent. A plus or minus range of 10 to 15 per cent occurs in some groups of dogs, however, under apparently constant environmental conditions. The *total pigment output* showed a +18 per cent increase on the average; it was increased or not altered in all but three tests, which is not a significant increase according to our previous observations[7, 8] on this type of biliary fistula preparation, in which a variation of + or −25 per cent of the mean may occur. A definite increase in *cholesterol output* occurred. The output of cholesterol, however, is increased when a choleresis is produced by the administration of conjugated ox bile acids or the return of dog's bile. According to our observations,[7] the cholesterol output varies + or −20 per cent under constant environmental conditions.

From 61 to 75 per cent of the cinchophen administered was recovered in the twenty-four-hour volume of bile. This agrees with the observations of Bradley and Ivy,[2] who recovered from 59 to 78 per cent in semiacute biliary fistula dogs. The test for cinchophen was negative in the precontrol and postcontrol cinchophen period.

Comment: It is clear from the results that cinchophen administered in 0.5 Gm. doses with the meals to the 5 dogs used produced no evidence of toxicity, using volume output, cholic acid synthesis, pigment, and cholesterol output as criteria. It is possible, however, that a period of three days of administration is not sufficiently long to produce hepatotoxic signs.

B. The Effect of Cinchophen Administration for Fourteen Days.—Following the same procedure as in the experiment above, 1 Gm. of cinchophen was given daily for fourteen days.

Dog 5 had suffered a partial obstruction of the bile duct several days prior to the start of the control period. We decided to use the animal to ascertain what might happen when cinchophen is administered during a time that the liver is recovering from an obstruction. During the obstruction the bile had a bad odor and was infected. This tended to clear when the obstruction was relieved.

TABLE II

THE EFFECT OF 1 GM. OF CINCHOPHEN DAILY FOR FOURTEEN CONSECUTIVE DAYS ON CHRONIC BILIARY FISTULA DOGS, NO BILE OR BILE SALT BEING GIVEN

DOG NO.	WT. (KG.)	VOLUME (C.C./24 HR.)			CHOLIC ACID (MG./24 HR.)			CHOLESTEROL (MG./24 HR.)			PIGMENT (MG./24 HR.)		
		C	T	% CHG.	C	T	% CHG.	C	T	% CHG.	C	T	% CHG.
2	10.0	129	232	+ 80	1,184	1,152	− 2	14	20	+42	114	136	+ 9
2′	11.0	129	291	+125	1,380	1,175	−14	13	18	+39	107	139	+30
S3	9.0	99	230	+132	1,455	1,601	+10	16	23	+44	126	120	− 4
4	8.4	127	322	+154	1,468	1,541	+ 5	16	29	+81	157	200	+27
5*	11.1	134	255	+ 90	1,106	718	−35	18	27	+50	111	122	+ 9
S3†	9.0	94	219	+133	1,199	1,289	+ 7	16	19	+18	128	143	+11
Average		119	258	+116	1,295	1,246	− 3	15	23	+50	124	127	+ 2

*See Table III.
†Cinchophen given for thirty-three days.

The results obtained from six experiments on the 5 dogs are shown in Table II. The results are approximately the same as those obtained in the three-day experiment.

In Dog 83, the second test on the dog recorded in Table II, cinchophen was administered for thirty-three days. During this period the animal consumed all his diet with appetite, though no bile was returned or bile salts were administered. The animals as well as the composition of the bile remained normal. The average volume output during the last eleven days was 254 c.c. per day, and the average cholic acid synthesis was 1,336 mg; both tended to be higher than during the earlier part of the experiment. It should be remarked that other dogs may synthesize and excrete less cholic acid during the first than during the second week.

TABLE III

THE EFFECT OF 1 GM. OF CINCHOPHEN DAILY IN THE PRESENCE OF LIVER DISEASE*

DOG. NO.	WT. (KG.)	DATE	POSTOP. DAY	HR. FLOW	DIET	VOLUME (C.C.)	CHOLIC ACID (MG.)	CHOLESTEROL (MG.)	PIGMENT (MG.)
5	11.1	12/28/40	101	24	Reg.	214	421		99.1
		12/29/40	102	24	Reg.	223	580		103.2
		12/30/40	103	24	Reg.	247	370	16.3	134.1
		12/31/40	104	24	Reg.	249	598		148.1
		1/ 1/41	105	24	Reg.	261	548		157.9
		1/ 2/41	106	24	Reg.	258	564		118.7
		1/ 3/41	107	24	Reg.	265	815		116.7
		1/ 4/41	108	24	Reg.	262	838	38.2	161.1
		1/ 5/41	109	24	Reg.	259	958		170.4
		1/ 6/41	110	24	Reg.	277	865		101.4
		1/ 7/41	111	24	Reg.	286	972	22.6	123.3
		1/ 8/41	112	24	Reg.	238	857		94.0
		1/ 9/41	113	24	Reg.	260	832		88.6
		1/10/41	114	24	Reg.	277	837	29.9	87.2
Average						255	718	27.0	121.7

*An obstruction of the bile duct was relieved three days before the start of this experiment. The low cholic acid output during the first few days is attributable to an hepatitis. The animal consumed all the diet each day.

No bile was returned; no bile salts were given.

The results on Dog 5 are of particular interest because the dog had not fully recovered from a partial biliary obstruction and hepatitis when cinchophen medication was started. The daily determinations are recorded in Table III. At the start of the experiment only 0.3 to 0.4 Gm. of cholic acid was synthesized daily. Later, cholic acid output increased to 0.8 to 0.9 Gm. daily.

Comment: On the basis of the criteria used, the administration of 1 Gm. of cinchophen daily, 0.5 Gm. with each meal, for fourteen days and in one case for thirty-three days caused no evidence of hepatic injury.

In the dog recovered from partial obstruction and hepatitis, the liver manifested a gradual recovery. Whether the recovery of the animal was retarded cannot be stated, since such animals in our experience may recover in from three to fourteen days when no cinchophen is given. However, in such instances we always give a bile salt preparation or return bile with the idea of flushing the ducts, since obstruction, as a rule, occurs in our dogs only when they are kept on food without bile salts or the return of bile. The thick bile formed in the absence of the return of bile or bile salts definitely favors obstruction and infection

of the bile; this is the chief reason why we never deprive our dogs of bile for a longer period than from five to seven days, except when we give them some other choleretic.

The fact that no cinchophen could be found in the bile twenty-four hours after the last dose was given shows that it is rather rapidly excreted or metabolized. The fact that we were able to obtain from 61 to 75 per cent of the administered cinchophen in the bile shows that cinchophen in the presence of an intact biliary tract may undergo repeated enterohepatic circulation. Since in our dogs the cinchophen given could undergo only one enterohepatic circulation, *it is possible that hepatic injury may occur in the intact animal due to repeated enterohepatic circulation.* This hypothesis was tested by the following experiment.

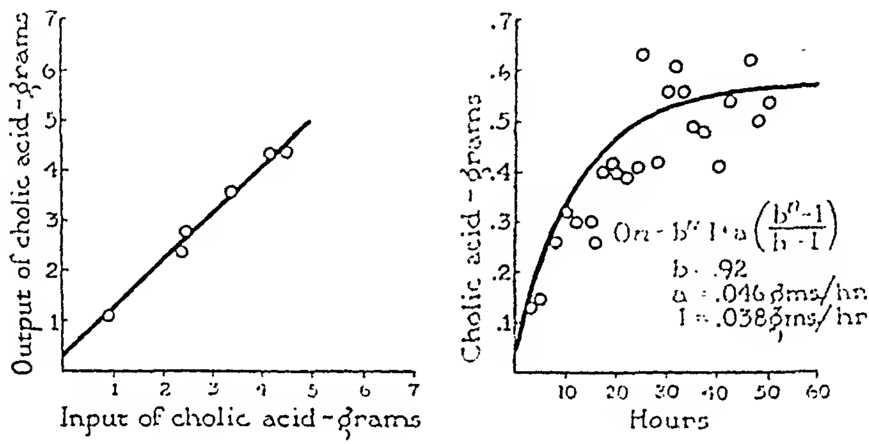


Fig. 1.

C. *The Effect of Repeated Enterohepatic Circulation of Cinchophen in the Dog.—Method.* One biliary-duodenal fistula "suction" dog[9] was used. The dog weighed 9.0 kg. The bile was collected in a glass cylinder and could be returned directly into the intestine through the duodenal fistula. The animal was fed at eight-hour intervals one-third of his daily ration of the standard diet. The bile was collected and returned on an hourly schedule, the volume being recorded and a 0.1 c.c. sample being retained for cholic acid assay. The bile collected hourly was returned to the intestine during a five- to ten-minute period for forty-eight hours. After a three-day control period of no return of bile the hourly return was started and 333 mg. of cinchophen was mixed with each eight-hour feeding.

Results. It has been shown previously[10, 11] that when bile is returned to an animal every hour after a period during which no bile has been returned, the cholic acid output, due to enterohepatic circulation, will increase according to a definite curve or formula and will in time reach a relatively constant or homeostatic level, at which synthesis is balanced by the "destruction" of cholic acid. In view of this fact, it was thought that if cinchophen disturbed cholic acid synthesis, the disturbance would result in a modification of the "normal" curve of accumulation. Therefore, the data obtained on cholic acid output on the hourly return of bile with cinchophen administration was treated mathematically as in previous work.[10, 11]

When the input of cholic acid is plotted against output of cholic acid, a linear relationship occurs (Fig. 1). When the results are analyzed mathematically and the equation of the straight line, output = "b" times input plus "a," was solved, it was found that "b," the slope of the line or per cent of recovery was 92 per cent, and "a," the point of intercept on the ordinate, or basal cholic acid output on the diet, was 0.046 Gm. per hour. The curve that fitted this straight line was predicted by the following equation: $On = b^nI + a\left(\frac{b^n - 1}{b - 1}\right)$, where "On" is the output of cholic acid at any subsequent hourly period, and "I" is the actual initial dose of cholic acid administered at the first return of bile. The curve predicted by this equation, which has been tested and established by previous experimental results,[10, 11] is shown in Fig. 1. The predicted homeostatic level was 0.60 Gm. per hour and that actually obtained experimentally was 0.55 Gm. per hour. The predicted cholic acid synthesis per hour was 0.046; the actual synthesis found was 0.050 Gm. The predicted recovery was 92 per cent, the actual was 91 per cent. The actual curve of output (Fig. 1) approximates fairly closely the predicted curve, though it is somewhat lower at the first, which is, in general, true in other such tests when cinchophen is not given.

Comment: The results show definitely, insofar as this dog is concerned, that repeated enterohepatic circulation of cinchophen during a forty-eight-hour period causes no decrease in cholic acid output.

DISCUSSION

The effect of cinchophen feeding on the secretion of bile has been studied under various conditions: (a) when fed for three-day periods; (b) when fed over a period of fourteen consecutive days, and thirty-three days in one animal; and (c) when making 24 enterohepatic circulations a day for two days. In no case were we able to show that cinchophen acted like a hepatotoxin as determined by bile analyses. The cholic acid output, which we believe is the most sensitive indicator of liver dysfunction, was not altered significantly. This was especially true of the average results on a number of biliary fistula dogs, although individual tests may show a greater variability in cholate synthesis. In all cases due to choleresis the cholic acid concentration in the bile was decreased, but the total cholate output remained on the average the same as the control. Similar results were obtained using potent oxidized bile acid choleretics.[7] It was also shown that cinchophen does not affect the regulation of cholic acid output by the liver. When the bile, containing cinchophen, was returned every hour during the day, the cholate output gradually increased and reached the homeostatic level predicted by a methematical equation.

We have no explanation for the increased cholesterol output due to cinchophen feeding; this occurs whenever a choleresis is produced by any procedure known. Since the cholic acid output was not simultaneously increased, the bile acid: cholesterol ratio would be decreased, which *may* tend to predispose to a precipitation of fatty acid and cholesterol in the gall bladder in the presence of a condition favoring the diffusion of bile salts from the organ.

These experiments establish cinchophen as a potent hydrocholeretic even as effective as dehydrocholic acid (decholin) or mixed ketocholanates (ketochol).

When 3 Gm. of these bile acids were administered per day, a +103 per cent increase, on the average, in volume output was obtained. One gram of cinchophen daily, whether administered over a short or long period of time, gave an average increase of +106 per cent. Comparing these substances on a gram-weight basis, cinchophen is three times more effective than dehydrocholic acid as a hydrocholeretic. The reason for this marked choleresis is not definitely known. In acute and chronic biliary fistula dogs the choleresis produced by cinchophen was associated with the excretion of 60 to 75 per cent of the administered drug. When 10 to 50 mg. of cinchophen were injected intravenously into acute and chronic biliary fistula rabbits, no hydrocholeresis occurred, and only very small amounts of cinchophen were excreted in the bile.[4] These results indicate that in the dog, but not in the rabbit, the liver is especially concerned in the excretion of cinchophen and in the process of its excretion water is excreted with the drug. It is quite probable that the ease of solubility of cinchophen in bile and its osmotic activity therein are important factors contributing to the hydrocholeresis produced by cinchophen administration in the dog.

SUMMARY AND CONCLUSIONS

One gram of "pure" cinchophen (Calco Company) was fed daily (0.5 Gm. with each meal) to 5 chronic biliary fistula dogs for three- and fourteen-day periods, and in one instance for thirty-three days, and the effect on bile volume, cholic acid, total pigment, and cholesterol outputs was determined. The amount of cinchophen recovered in the bile was also determined. It was found that this daily dose of cinchophen caused a marked hydrocholeresis associated with no significant change in cholic acid and total pigment outputs, a marked increase in cholesterol output, and a recovery of 60 to 75 per cent of the administered cinchophen.

On a gram-weight basis cinchophen is three times as effective as a hydrocholeretic as the most potent bile acid choleretic, namely, dehydrocholic acid.

When bile, formed under the stimulus of cinchophen feeding, is returned every hour during the day for two days to simulate conditions in the intact animal and to cause repeated enterohepatic circulation of the cinchophen and bile salts, the cholic acid regulatory mechanism was not disturbed and the cholate output increased and closely approximated the predicted homeostatic level of the nonmedicated animal.

In the presence of an already injured liver, cinchophen feeding did not definitely increase the severity of the hepatitis; it cannot be said that the cinchophen aided recovery, though recovery occurred during cinchophen medication.

Therefore, from our studies on chronic biliary fistula dogs, cinchophen, when fed in 1 Gm. daily doses, does not seem to act as a hepatotoxin on the basis of the criteria used. To what extent the "margin of safety" in the liver was affected by the cinchophen is not clearly determined by the type of experiment used in these studies.

REFERENCES

1. Brugsch, T., and Horsters, H.: Ztschr. ges. Exper. Med. 38: 367, 1923.
2. Bradley, W. B., and Ivy, A. C.: Proc. Soc. Exper. Biol. & Med. 45: 143, 1940.
3. Stransky, E.: Biochem. Ztschr. 155: 256, 1925.
4. Berman, A. L., and Ivy, J. H.: Proc. Soc. Exper. Biol. & Med. 45: 853, 1940.

5. Goodman and Gilman: The Pharmacological Basis of Therapeutics, New York, 1940.
6. Rous, P., and McMaster, P. D.: J. Exper. Med. 37: 11, 1923.
7. Berman, A. L., Snapp, E., Ivy, A. C., Atkinson, A. J., and Hough, V. H.: Am. J. Digest. Dis. 7: 333, 1940.
8. Schmidt, C. R., Beazell, J. M., Berman, A. L., Ivy, A. C., and Atkinson, A. J.: Am. J. Physiol. 126: 120, 1939.
9. Kocour, E. J., and Ivy, A. C.: Am. J. Physiol. 122: 325, 1938.
10. Berman, A. L., Snapp, E., Ivy, A. C., and Atkinson, A. J.: Am. J. Physiol. 131: 776, 1941.
11. Berman, A. L., Snapp, E., and Ivy, A. C.: Proc. Am. J. Physiol., April, 1941.
12. Davis, M. W., Bradley, W. B., Bachrach, W. H., and Ivy, A. C.: Proc. Soc. Exper. Biol. & Med. 45: 66, 1940.

DRUG PROPHYLAXIS AGAINST LETHAL EFFECTS OF SEVERE ANOXIA

IV. A Standardized Technique

Influence of Body Weight, Injection of Saline, Muscular Restraint, Rate of Ascent, and Pretreatment With Oxygen or Helium-Oxygen in Anoxic Mice

G. A. Emerson, Ph.D., and E. J. Van Liere, Ph.D., M.D.
Morgantown, W. Va.

THE influence of several medicinal agents upon resistance to acute anoxia has been studied by numerous investigators, and their reports are summarized in the first paper of this series.[1] Major variations of technique and the lack of critical examination of the findings of different workers may be responsible for discordant conclusions regarding individual agents. It is difficult to make any adequate correlation of prophylactic effects of different agents when results obtained under widely different experimental conditions are compared. Relative efficacy of different agents may be estimated accurately only through the use of one or more standard techniques, and a concept of the relative involvement of the various physiologic processes known to be concerned in the lethal action of anoxia may then be gained.

Factors of simplicity, inexpensiveness, and uniform reproducibility are of primary importance in a test of prophylactic effects against anoxia, and these considerations governed the development of the present method. Since a standard technique should involve a test of effects of agents in intact mammals, with simultaneous observation of lethal effects of anoxia in sufficient numbers of treated and untreated animals to permit statistical evaluation of results, mice are best suited for use in the proposed method.

The most easily controlled type of anoxia is anoxic anoxia, as produced by exposure to low atmospheric pressures. In contrast, controlled degrees of anemic, histotoxic, or stagnant anoxia are extremely difficult, if not impossible, to obtain,

From the Departments of Pharmacology and Physiology, West Virginia University School of Medicine, Morgantown.
Received for publication, August 3, 1942.

especially in large groups of animals. Further, anoxic anoxia is probably more commonly encountered in diseases of man than are the other types. It may be emphasized, however, that results of tests upon small mammals and upon lethal effects of acute exposure to simulated high altitudes necessarily have no immediate applicability to the treatment of the moderate degrees of anoxia occurring in many diseases of man or in modern aviation practice. The function of a simple test of the antagonistic action of different agents toward lethal effects of anoxia in animals is one of orientation, after which more intensive study may suggest the possible clinical usefulness of those agents which show promise in examination by more elaborate techniques. In some instances, agents which are not prophylactic against, or are even synergistic with the lethal effects of exposure to simulated high altitudes may, nevertheless, be useful in the treatment of effects of moderate degrees of anoxia. Recognition of the limitations of such agents in the treatment of severe anoxia is still necessary for full knowledge of their action.

The scope of the problem concerning various agents other than oxygen, which may have protective value in severe anoxia, has been presented elsewhere.[1] The prophylactic effects of certain convulsants,[1] narcotics,[2] and of the optical isomers of benzedrine[3] have been studied by the technique described below.

METHOD

The method involves the use of sufficiently large numbers of mice for statistical significance, simultaneous exposure of an untreated control group of equal size to the same conditions of anoxia, and a comparison by statistical criteria[4] of mortality in control and treated groups at the time approximately 50 per cent of the control mice have died from anoxia.

Young healthy adult mice of the same strain are weighed to the nearest gram and are treated intraperitoneally with the test agent dissolved in saline. Control mice are treated with an equal volume of saline at the same time. This step is unnecessary under ordinary conditions but is desirable since it makes the degree of hydration more uniform in all mice. The interval between treatment and exosure to anoxia may vary with different agents according to their time of maximum action, but, in general, should be at least one hour for the longer-acting agents to minimize effects of the difference in time of treatment of the first and last mice injected.

Exposure to anoxia is produced in a cylindrical decompression chamber resembling that of Kolls and Loevenhart.[5] This tank is constructed of ¼ inch boiler plate, oxyacetylene-welded at all seams. Its horizontal diameter is 90 cm. and its height 45 cm. The capacity is approximately 285 liter. The tank is provided with three double-strength plate glass windows, reinforced with metal bars, to permit illumination and observation; a U-shaped mercury manometer with one arm open to the outside atmosphere; two adjustable inlet valves set oppositely at the top; and an outlet connected with a vacuum pump of high capacity. Ventilation is adequate in all parts of the tank at the lowest pressures used with biologic material, and there is no possibility of accumulation of carbon dioxide under the conditions of the proposed test.

Although it is recognized that no valid single degree or even moderately narrow range of anoxia can be taken as the point at which 50 per cent of control mice die,[1] the conditions of producing anoxia should be kept rigorously constant. The varying resistance of different control groups to anoxia then probably reflects constitutional differences, for there is no correlation with the pre-experimental barometric pressure, humidity, or apparent physiologic condition of the mice. This variation in tolerance on different days necessitates the simultaneous exposure of treated and control groups to identical conditions.

Mice within the tank are rapidly exposed to a reduced pressure of about 523 mm. Hg, which simulates an altitude of approximately 10,000 feet. This pressure is maintained for ten minutes. After this period of equilibration of blood and tissue gases with this level of reduced pressure, the pressure is further reduced at a rate corresponding to an ascent of 1,000 feet per minute. Mice usually tolerate this progressive anoxia until a reduced pressure of less than 226 mm. Hg is attained within some thirty minutes after beginning exposure; this pressure corresponds to that at an altitude of 30,000 feet. Approximately 50 per cent of control mice die at the time atmospheric pressures of 170 to 141 mm. Hg are attained, which correspond to simulated altitudes of 36,000 to 40,000 feet.

Termination of exposure is governed by mortality of 50 per cent of the control mice rather than by attainment of a fixed level of anoxia. Mortality of 50 per cent of the control group affords the most significant point for statistical comparison of mortality in treated groups and estimation of either protective or harmful effects of the different agents. An ideal prophylactic would completely prevent lethal effects of anoxia at a level causing 100 per cent mortality of control mice. Except oxygen, no agent has yet been found capable of this.

After exposure, mice are observed for an additional forty-eight hours to include any possible late effects of treatment. Latent lethal effects have not been encountered with the agents tested thus far. Necropsies are occasionally made of mice dying during exposure to anoxia. Groups of mice showing pneumonitis should not be used.

The influence of diet, and especially of various hypervitaminoses, is to be studied in detail. As the test has been used to date, mice have been supplied with a standard diet and water, available at all times. The mice have been subjected to anoxia in the early afternoon. Since mice are chiefly nocturnal feeders, the stomach and small intestine are usually fairly empty at this time and the mice are in a postabsorptive state.

BODY WEIGHT

Immature animals are less susceptible to lethal effects of anoxia than are adults,[6, 7] and smaller species of animals are less susceptible to aeroembolism than are larger species.[8] In a test of tolerance to anoxia the validity of results obtained with unselected groups of young adult mice may be questioned. If the smaller mice of such groups are significantly less susceptible to anoxia, and if the numbers of smaller mice in control and treated groups do not coincide, errors might arise from this cause. Data have accumulated from previous studies which bear upon this problem.

The mean body weight of 160 mice dying during exposure for thirty minutes to severe anoxia was 16.8 ± 0.21 Gm., while the mean body weight of 240 mice surviving the same conditions of anoxia was 16.1 ± 0.17 Gm. The difference between these means is 0.7 ± 0.27, signifying[7] a p of <0.01. However, this difference is more apparent than real, for the data were integrated from results with several groups of mice of different mean body weights and of differing tolerance to anoxia, as noted by differences in mortality ratios in the individual experiments. The statistical treatment of such data cannot be considered conclusive. With smaller groups[2] simultaneously exposed and, therefore better controlled, the differences between mean body weights of mice surviving and those dying under identical conditions of anoxia were not significant, although the mice dying during anoxia were uniformly slightly heavier than those simultaneously surviving.

Of 100 mice from the same colony, 52 survived and 48 died when subjected simultaneously to severe anoxia produced according to the proposed technique. Mean body weights of these two groups were 15.6 ± 0.47 and 16.0 ± 0.40 Gm., respectively. The difference between these means is not significant, although again the survivors were slightly lighter than those dying in anoxia.

Neonatal tolerance of the rat[9] to anoxia is rapidly lost within eighteen days of postnatal life. It does not appear to be necessary that young adult mice should be carefully selected within a narrow weight range for use in tests of tolerance to anoxia. It may be noted that even if the coefficient of correlation of body weight and tolerance to anoxia were a significantly large negative value, the source of error from this cause in the proposed technique would still be dependent upon inequalities in numbers of small mice in control and treated groups. The use of large groups of mice minimizes this hazard.

INJECTION OF SALINE

It was previously reported[1] that intraperitoneal injection of 50 c.c. per kilogram of physiologic saline fifteen minutes before exposure to anoxia has no harmful effect on tolerance of mice. Thirteen of 20 mice so treated died under anoxic conditions simultaneously causing death of 14 of 20 untreated mice. Since then mice used as controls have been treated routinely with saline injections in amounts as great as 60 c.c. per kilogram giving one hour before exposure, without apparent effect on tolerance. If appreciable hydremia is produced by these massive doses of saline, the resultant anemia from dilution of the blood is insufficient to affect tolerance to anoxia. If blood volume is actually increased by these saline injections, an undesirable accentuation of the cardiac dilatation known to occur in anoxia[10] would probably outweigh any possible beneficial effects. Whatever the circulatory effects of massive doses of saline may be, however, they do not appreciably affect the lethal action of severe anoxia under the conditions of the proposed test.

MUSCULAR RESTRAINT

Mice become hyperactive during induction of anoxia, more or less in proportion to the rate of production of anoxia. In susceptible mice, and in the majority after administration of subconvulsive doses of central nervous system stimulants,[1]

this hyperactivity leads to severe clonic or epileptiform convulsions. Mice exhibiting these convulsions usually become comatose and die within a short period. Typical tetanic convulsions are infrequent in anoxic mice, and the cause of the usual convulsion in anoxia is probably cortical and sympathetic stimulation, or depression of the motor inhibitory mechanisms, rather than nervous effects of the alkalosis due to hyperpnea in early anoxia. The possibility that the hyperactivity is a response to painful stimuli from aeroemboli in joints or other tissues is negated by findings which indicate that aeroembolism is not an appreciable factor under the experimental conditions. Distention of hollow viscera due to expansion of contained gas under reduced pressure could, however, give rise to strong pain stimuli, and neuralgic pain arising from local effects on nerve trunks may occur in mice as in man.[8]

The metabolic rate is much higher during active exercise than in quiescence, and tolerance to anoxia should thereby be decreased. A significant increase in tolerance to anoxia is shown by mice fully narcotized with certain depressants[2] over that of control mice with normal activity. In order to test whether simple physical restaint would produce like results, the following two experiments were made.

Ten mice were confined, each within a 60 c.c. beaker covered with a single layer of cotton gauze. Under anoxic conditions in which 18 of 40 normal mice died when simultaneously exposed and allowed to run about inside the decompression chamber, all the 10 mice confined in the beakers died. It was felt that perhaps adequate ventilation was prevented and also that the confinement was not sufficient to restrain the mice from struggling, although none showed frank convulsions.

Twenty mice were therefore confined, each within a tube of light 14-mesh wire gauze. The tubes were made of a single thickness of the wire gauze, closed by pinching the ends and loosely stitching the longitudinal seam with light copper wire. Each tube was constructed to fit the occupant snugly but to prevent major movements of the body and full extension of the limbs. The mice were free to move easily forward along the tube but could not turn around. The usual response to confinement was sleep. Special care was taken not to make the tube too small for full respiratory movements, and the longitudinal closure was loose enough to permit a considerable amount of enlargement of the lumen with little resistance, to correct for any moderate increase in body volume at reduced pressures.

Of the 20 mice thus restrained, all died under conditions of anoxia in which only 9 of 20 control mice died when allowed the freedom of the decompression chamber.

In both experiments the rate of production of anoxia conformed to that noted in the proposed method, with a plateau for ten minutes at a simulated altitude of 10,000 feet, followed by a simulated rate of ascent of 1,000 feet per minute to 41,000 feet. With this relatively slow rate of ascent, mice show a minimal amount of excitement, although convulsions commonly precede death at the higher levels of anoxia. It may be concluded that, in testing prophylactic drugs by the proposed technique, close confinement of the experimental animals is neither beneficial nor desirable and does not resolve irregularities in oxygen

consumption due to differing activities of the mice as efficiently as does the use of a rate of production of anoxia which minimizes excitement. The harmful effects of restraint may be due to marked isometric muscular activity resulting in an increase in oxygen consumption actually greater than that in mice allowed to run about at will, and perhaps also to increased excitement and sympathicoadrenal stimulation consequent to confinement.

RATE OF ASCENT

Tolerance to anoxia is related to the rate of production of anoxia in two major ways: through development of compensatory mechanisms of the organism and through cumulative effects of prolonged anoxia. When animals are very rapidly exposed to low atmospheric pressures, limitations of the rate at which the body can lose oxygen and the possibility of occurrence of aeroembolism are also involved. Heim and Armstrong[11] have reported an increase of about 50 per cent in altitude tolerance as the rate of ascent is increased from 100 to 30,000 feet per minute. They believe that effects of anoxia upon the central nervous system are greatest with rapid production of anoxia, and that cardiovascular effects are greatest with slow ascents. It would follow that different types of drugs might exhibit different degrees of prophylaxis according to the rate of production of anoxia. Tests of lethal effects by different techniques of producing anoxic anoxia are of interest as an index of the possibility of aeroembolism. Dilution techniques[12] completely avoid any possibility of aeroemoblism but present certain technical difficulties. Anoxia was, therefore, produced in the decompression chamber at five different simulated rates of ascent and lethal effects of these different treatments were observed.

Five groups of 20 mice each were subjected to anoxia produced at one of the rates noted in Fig. 1. Curve A represents a simulated rate of ascent of approximately 15,000 feet per minute until an approximate altitude of 37,000 feet was attained, followed by a slow ascent of about 100 feet per minute for ten minutes. Approximately one-half of the mice died at a simulated altitude of 37,500 feet, six minutes after beginning exposure to anoxia. Curve B represents a simulated rate of ascent of about 1,000 feet per minute. One-half of the mice died at a pressure corresponding to 42,000 feet, thirty-eight minutes after beginning exposure to anoxia. Curves C and D represent rates of ascent coinciding with that of curve A until approximate altitudes of 11,000 and 21,500 feet, respectively, were attained. These levels of anoxia were then maintained for ten minutes, after which a further simulated rate of ascent of approximately 2,000 feet per minute was begun. One-half of the mice so treated died at a simulated altitude of 37,000 feet twenty-three minutes after beginning exposure, and at a simulated altitude of 42,000 feet twenty minutes after beginning exposure, for treatments represented by curves C and D, respectively. Curve E represents rapid ascent to about 32,000 feet, maintenance of this level for ten minutes, and a further ascent at the rate of about 1,000 feet per minute. One-half of the mice treated in this way died at an approximate altitude of 32,000 feet, nine minutes after beginning exposure to anoxia.

Curves A, D, and E terminate at the level at which the last animal of the group died. Two of the 20 mice subjected to the treatment represented by

curve C recovered after exposure, despite brief apnea in both. Seven of the 20 mice subjected to the slow rate of ascent represented by curve B recovered after exposure. Comparison of the minimal pressures attained in the different treatments is meaningless, since these pressures reflect the tolerance of only the most resistant mice in each group. A more significant comparison is obtained by consideration of the levels of anoxia at which 50 per cent of the mice died.

The above tests were made consecutively, under identical conditions of barometric pressure, humidity, and temperature, and all mice were of the same strain with identical pre-experimental care and diet. The results indicate that aeroembolism is not a significant factor as a cause of death under the conditions of the proposed test. After a return of mice to room conditions, even those subjected to the most severe conditions noted in Fig. 1 showed no gaseous emboli in vessels of the pia mater or in the abdomen.

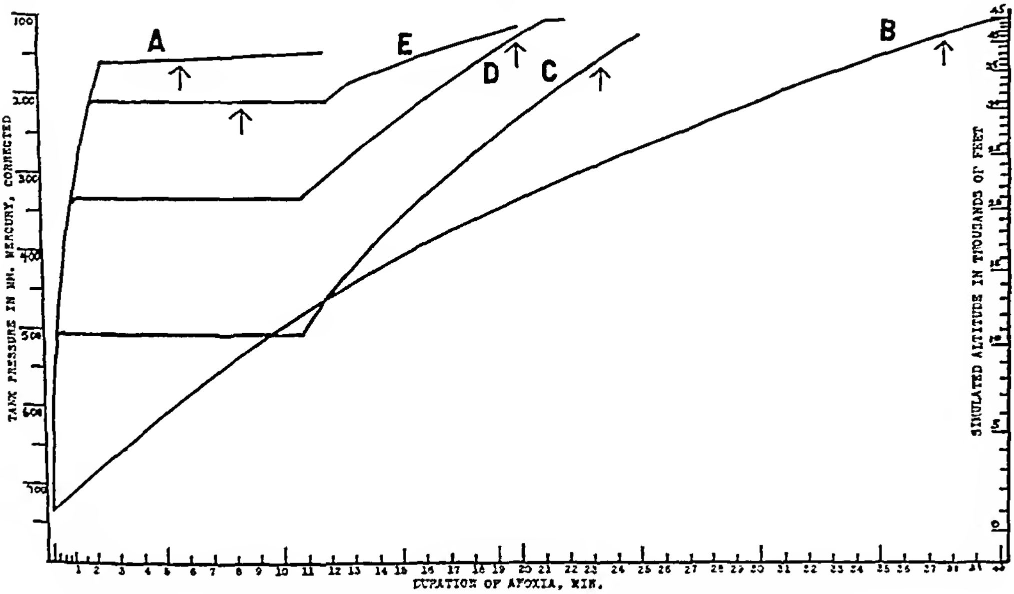


Fig. 1.—Effect of simulated rate of ascent on tolerance of mice toward lethal effects of anoxia ↑ denotes point at which one-half of the mice died.

The results also indicate that a simulated rate of ascent of 1,000 feet per minute is slow enough to permit various compensatory changes in mice but is rapid enough to avoid any marked cumulative effects of anoxia.

PRETREATMENT WITH OXYGEN

Thirty mice were placed in an 8 liter sealed glass chamber containing 1 kg. of soda lime so placed that the mice did not have access to it. Oxygen was passed through the chamber at a rate of 2 liters per minute for ninety minutes. The mice were then placed in the decompression chamber together with 30 mice which had been kept under normal atmospheric conditions. The pressure was quickly reduced to a simulated altitude of 20,000 feet and was held at this level for ten minutes. The pressure was then further reduced at a rate simulating an ascent of 2,000 feet per minute until an altitude of about 38,000 feet was attained, and this degree of anoxia was then maintained. The total time of exposure was thirty minutes.

Twenty-one of the 30 previously untreated mice died from anoxia, while only 14 of the 30 mice which received oxygen prior to exposure died. Although this difference in mortality is large, it cannot be considered as a statistically significant difference since its p is greater than 0.05.

The rate at which oxygen was supplied during the pretreatment period is equivalent to a complete change of the atmosphere within the chamber every 3 to 3.5 minutes, or a total of 26 to 30 complete changes in ninety minutes. Under these conditions appreciable denitrogenation of the tissues occurs. The significance of this in regard to aeroembolism is discussed below. Consideration of the factors involved in this experiment led to observation of effects of pretreatment with helium-oxygen.

PRETREATMENT WITH HELIUM-OXYGEN

Thirty mice were treated in the same way as in the pretreatment with oxygen alone, except that pure oxygen was passed through the chamber at the rate of 2 liters per minute for only thirty minutes. The chamber was then rapidly flushed with 18 liters of helium, quickly followed by 30 liters of a mixture of 80 per cent helium and 20 per cent oxygen. The outlet tube from the chamber was closed and the chamber was connected with a reservoir of pure oxygen maintained at atmospheric pressure, thus permitting the flow of oxygen at the rate at which the soda lime within the chamber absorbed carbon dioxide excreted by the mice. Mice were kept in this atmosphere of 80 per cent helium and 20 per cent oxygen for one hour, during which period the chamber was flushed three times with 80 per cent helium and 20 per cent oxygen in order to remove any accumulated nitrogen.

The mice were then subjected to anoxia in the decompression chamber, together with 30 previously untreated mice. The procedure of producing anoxia was the same as with mice pretreated with oxygen alone.

Of the 30 control mice, 11 died during the exposure to anoxia while only 6 of the 30 mice pretreated with helium-oxygen died. Again this difference in mortality is large, but it is not statistically significant. The implications of these data are discussed below.

DISCUSSION

Factors which may influence results in the proposed test have been mentioned, and their significance has been appraised experimentally. Body weight of young adult mice is not correlated significantly with resistance to anoxia. Therefore, groups of young adult mice need not be carefully selected within a narrow weight range for use in the test. Massive intraperitoneal injections of saline have little effect on tolerance to anoxia, so that control groups need not receive amounts of saline equivalent to those given as a solvent for the test agents, although this treatment with placebo injections in control mice is probably desirable to render the degree of hydration more uniform in all mice. Muscular restraint, as produced by two methods, fails to increase tolerance of mice to simulated high altitudes and appears markedly detrimental, at least under conditions of decompression which minimize excitement in mice allowed the freedom of the chamber.

The remaining factors involve the important question of the possibility of occurrence of aeroembolism in mice subjected to the proposed experimental technique. If an appreciable number of deaths under the experimental conditions can be ascribed to aeroembolism rather than to anoxia, these conditions obviously cannot yield a significant measure of prophylactic actions of drugs against lethal effects of anoxia.

Effects of decreased atmospheric pressures upon the blood and spinal fluid have been studied intensively.[8, 13] Armstrong[8] has pointed out that in man an acute exposure to decreased atmospheric pressure, such as might occur in sudden failure of a sealed cabin in aircraft at high altitudes, would result in anoxia at altitudes below 30,000 feet, in aeroembolism at altitudes between 30,000 and 63,000 feet, and in rapid vaporization of body fluids at altitudes above 63,000 feet. In experimental observations, however, he[8] found that while goats may show the presence of nitrogen bubbles in blood during exposure to high altitudes, this phenomenon is not apparent in rabbits subjected to a simulated rate of ascent of 40,000 feet in forty seconds. With goats nitrogen bubbles appear in the spinal fluid at a simulated altitude of 18,000 feet, and increase as ascent is continued. Intracranial pressure simultaneously increases.

Walsh and Boothby[13] investigated effects of low atmospheric pressure on spinal fluid in man and found that exposure to simulated altitudes as low as 10,000 to 12,000 feet elicits the appearance of fine bubbles "like those arising from champagne" and that these increase in size and number until an altitude of 28,000 feet is attained. On maintenance of this level for five minutes, the bubbles disappear. Intraspinal pressure increases progressively by some 3 cm. after the appearance of bubbles. Similar findings were observed in another subject partially denitrogenated by prior exposure to an atmosphere of pure oxygen. In vitro tests substantiated these results. With a monkey, however, a roentgenogram did not disclose the presence of any accumulation of gas in the ventricles of the brain even after a rapid ascent to a simulated altitude of 40,000 feet.

From these reports it may be concluded that smaller mammals withstand lower levels of atmospheric pressure before exhibiting formation of nitrogen bubbles in blood and spinal fluid. This characteristic is probably related to different rates of elimination of nitrogen due to differences in circulatory rates. Thus, in an atmosphere of pure oxygen, 95 per cent denitrogenation occurs within two hours in dogs and four hours in man, and the circulatory rate of the dog is about twice that of man, expressed in terms of blood supply per kilogram of body weight.[14] Extrapolation of results with rabbits and monkeys to mice appears justifiable, and the possibility of occurrence of aeroembolism in mice under the conditions of the proposed test would seem remote. Three points of evidence in the present study support this view and suggest that there is no hazard of aeroembolism under the experimental conditions. A further, though minor, confirmatory point involves the lack of a significantly demonstrable difference in tolerance in adult mice of different body weight: although there may be no valid relative difference in amount of fatty tissue in large and small mice, it is reasonable to assume a difference in absolute amount of body fat and fatty tissue acts as a reservoir for nitrogen, favoring aeroembolism.[6]

First, consideration of the mortality of mice subjected to different rates of production of decreased atmospheric pressure reveals that no effects definitely attributable to aeroembolism occur even with the most rapid rates of ascent. There is no sudden, large increase in lethal effects of exposure to low pressure other than the moderate influences related to failure of compensatory mechanisms antagonizing anoxia. However, to minimize further any possibility of aeroembolism, the proposed test utilizes a partial denitrogenation by exposure for ten minutes to a reduced pressure easily tolerated by the mice; i.e., a plateau at 10,000 feet. Absence of persistent aeroemboli in vessels of mice after drastic anoxic treatment, following return to normal atmospheric pressure, does not prove that aeroemboli do not occur at the height of anoxia, but all considerations indicate that aeroembolism is not an important factor even in mice subjected to rapid ascent.

Second, exposure to pure oxygen for ninety minutes before the mice are subjected to anoxia results in a significant denitrogenation of the tissues, probably of more than 95 per cent. Such treatment does not significantly reduce the lethal effects of anoxia as produced in the proposed test. Although there is no recognized reservoir for oxygen save the small effect of complete saturation of myohemoglobin,[12] minor physiologic effects of pretreatment with oxygen might be sufficient to explain the slightly increased tolerance of pretreated mice. Certainly there is not the major difference in mortality between pretreated and control mice which could be expected if aeroembolism were a major factor in the lethal effects of the conditions to which the mice are subjected in the proposed test.

Finally, pretreatment with helium-oxygen also does not significantly reduce lethal effects in mice. At atmospheric pressure helium is liberated from tissues about twice as fast as nitrogen and, therefore, should have less tendency to become supersaturated in blood and tissues during exposure to low pressures.[15] If aeroembolism were an important cause of death in the majority of the control mice exposed to the simulated high altitudes, pretreatment with helium-oxygen should significantly decrease this mortality.

It is to be noted that the possibility of an infrequent instance of aeroembolism occurring under the conditions of the proposed test is not ruled out conclusively by any one of the three factors discussed. Taken together, however, there is a strong indication that aeroembolism occurs so rarely, if at all, under these conditions that it does not invalidate interpretation of results obtained through a decompression technique as a true expression of tolerance of the mice to anoxia.

SUMMARY

A standard test of effects of drugs upon tolerance to anoxia is proposed. The method involves exposure of large groups of mice to low atmospheric pressures produced by a rigorously fixed technique in a decompression chamber allowing adequate ventilation, with simultaneous exposure of an equal number of untreated mice to the same conditions, and a comparison of mortality in control and treated groups by statistical criteria at the time 50 per cent of the control mice have died from anoxia. The function of this simple test is one of preliminary orientation, to obtain a measure of relative efficacies of different agents in com-

bating lethal effects of anoxia. For reasons discussed results cannot be considered applicable to therapy in man without further investigation. The influence of certain variable factors, including those which might affect the possible occurrence of aeroembolism, is studied. The results indicate that the test measures tolerance to anoxia and is not significantly affected by other factors.

REFERENCES

1. Emerson, G. A., and Van Liere, E. J.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. I. Convulsant Agents, Arch. Internat. de pharmacodyn. et de thérap. 64: 239, 1940.
2. Emerson, G. A., Van Liere, E. J., and Morrison, J. L.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. II. Alcohol, amytal and pentobarbital, Proc. Soc. Exper. Biol. & Med. 49: 376, 1942.
3. Emerson, G. A.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. III. *d*-, *l*-, and *dl*-Phenisoprophylamines, J. Pharmacol. & Exper. Therap. 76: 11, 1942.
4. Loewenthal, L. J. A., and Wilson, W. A.: Problems of Chance in Medicine and Research, Brit. M. J. 2: 110, 1939.
5. Kolls, A. C., and Loevenhart, A. S.: A Respiratory Chamber for Small Animals, Am. J. Physiol. 39: 67, 1915.
6. Kabat, H.: The Greater Resistance of Very Young Animals to Arrest of the Brain Circulation, Am. J. Physiol. 130: 588, 1940.
7. Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E.: Tolerance of the Newborn to Anoxia, Am. J. Physiol. 134: 281, 1941.
8. Armstrong, H. G.: Principles and Practice of Aviation Medicine, Baltimore, 1939, Williams & Wilkins.
9. Fisher, R. A.: Statistical Methods for Research Workers, ed. 7, Edinburgh, 1938, Boyd and Oliver.
10. Van Liere, E. J., Crisler, G., and Hall, J. E.: The Effect of Digitalis on Acute Cardiac Dilatation Produced by Anoxemia, J. Pharmacol. & Exper. Therap. 52: 408, 1934.
11. Armstrong, H. G., and Heim, J. W.: Factors Influencing Altitude Tolerance During Short Exposures to Decreased Barometric Pressures, J. Aviation Med. 9: 45, 1938.
12. Van Liere, E. J.: Anoxia, Its Effect on the Body, Chicago, 1942, University of Chicago Press.
13. Walsh, M. N., and Boothby, W. M.: The Demonstration of Air Bubbles in the Spinal Fluid Under Atmospheric Pressures, Produced in a Low Pressure Chamber, Approximating Those Obtaining During Rapid Ascents in Airplanes, Proc. Staff Meet., Mayo Clin. 16: 225, 1941.
14. Behnke, A. R.: The Application of Measurements of Nitrogen Elimination to the Problem of Decompressing Divers, U. S. Nav. M. Bull. 35: 219, 1937.
15. Behnke, A. R., and Yarbrough, O. D.: Physiologic Studies of Helium, U. S. Nav. M. Bull. 36: 542, 1938.

DRUG PROPHYLAXIS AGAINST LETHAL EFFECTS OF SEVERE ANOXIA

V. Agents Affecting the Autonomic Nervous System

G. A. Emerson, Ph.D., and E. J. Van Liere, Ph.D., M.D.
Morgantown, W. Va.

Effects of anoxic anoxia on the activity of the autonomic nervous system have been summarized recently by one of us.[1] The bulk of the literature indicates that moderate or severe anoxia increases activity of the thoracolumbar division and evokes a discharge of epinephrine from the adrenal glands. The influence of anoxia on the craniosacral division is not as clear, although a compensatory stimulation might be expected even in the absence of direct effects. Monge[2] described an increased activity of the oculocardiac reflex in anoxic subjects, and others whom he cites have claimed that effects of atropine are diminished at high altitudes. Ury and Gellhorn[3] noted that in rabbits the threshold for reflex mydriasis by stimulation of the sciatic nerve is increased in anoxia, which may be interpreted as an indication of increased inhibition through the oculomotor nerve. Gasser and Loevenhart[4] found the cardio-inhibitory center less susceptible to anoxia than the vasomotor center, both in the primary stimulation and subsequent depression. On the other hand, there is a controversy[1] as to whether tachycardia in anoxia is due to stimulation of the accelerator mechanism or to vagal depression. The recognized disturbance of sleep at high altitudes may perhaps indicate in part a preponderance of adrenergic over cholinergic stimulation by anoxia.

Since the work of Gomez and Pike,[5] several reports have emphasized the relative resistance of autonomic ganglia to structural damage by anoxia. Autonomic neurons are much less susceptible than the central nervous system. Thoracolumbar ganglia are more susceptible than Auerbach's plexus to loss of function in anemic anoxia. Medullary centers controlling autonomic activity are more susceptible than the ganglia, and presumably the higher "centers" are still more susceptible.

Cats deprived of the larger part of their autonomic nervous system quickly die from degrees of anoxia ordinarily sublethal.[6] Thyroidectomy, which is said[7] to reduce susceptibility to anoxia, decreases the effects of epinephrine and increases cholinergic responses[8] while hyperthyroidism has the opposite effects.

Binet and Strumza[9, 10] and a few other observers have made direct studies of effects of several sympathicomimetic amines at lethal levels of anoxia. These reports are considered in the discussion of the present results.

A comprehensive survey of prophylactic actions of representative autonomic agents has been lacking and was, therefore, undertaken as a part of a series of

From the Departments of Pharmacology and Physiology, West Virginia University School of Medicine, Morgantown.
Received for publication, August 3, 1942.

studies designed to yield a preliminary evaluation of effects of different classes of drugs in anoxia for direction of more intensive pharmacologic study without which no clinical application of the results should be made. Maximal effects were desired in the present work, and conclusions as to the effects of clinical doses in man are not necessarily justified.

METHOD

A standardized technique of quantifying actions of drugs on lethal effects of anoxic anoxia was used. This method has been studied critically.[11] In brief, it depends on the comparison of mortality in groups of treated and control mice simultaneously exposed to identical conditions of anoxia produced in a manner which, we believe, avoids all such factors as aeroembolism which would invalidate the results.

Forty-one agents acting on the autonomic system or closely related to agents which do so were tested with small groups of mice. In 14 instances results were sufficiently suggestive to justify study with larger groups. The agents are divided into six classes, five of which are noted in Tables I to V. The sixth group is that of adrenergic sensitizers, of which cocaine was taken as the sole example.

TABLE I

EFFECT OF SYMPATHETIC STIMULANTS AND RELATED AGENTS ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Phenethylamine hydrochloride	50	10/20	9/20
Phenethanolamine sulfate	50	8/10	5/10
l-Ephedrine hydrochloride	25	9/10	4/10
d-Pseudoephedrine hydrochloride	25	7/10	4/10
Hordenine sulfate	50	6/20	9/20
Tyramine (free base)	25	2/10	5/10
Propadrine hydrochloride	25	10/10	5/10
dl-Norephedrine hydrochloride	25	9/10	5/10
dl-Pseudonorephedrine hydrochloride	25	9/10	5/10
dl-Phenisopropylamine sulfate	10	50/50	11/50
	2	12/30	5/30
	0.2	18/30	9/30
l-Phenisopropylamine sulfate	10	47/50	11/50
	2	6/30	5/30
	0.2	15/30	9/30
d-Phenisopropylamine sulfate	10	50/50	11/50
	2	16/30	5/30
	0.2	11/30	9/30
Epinephrine hydrochloride	0.3	26/30	28/30
Mescaline hydrochloride	50	5/10	4/10
l-dihydroxyphenylalanine phosphate	100	7/10	5/10
Methylaminomethylheptene hydrochloride	50	9/10	5/10

TABLE II

EFFECTS OF SYMPATHICOLYTIC AGENTS ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Ergotamine tartrate	5	6/25	15/25
Yohimbine hydrochloride	5	3/10	6/10
	1	5/10	6/10
Diethylaminomethylbenzodioxane hydrochloride	10	5/25	15/25
Piperidinomethylbenzodioxane	10	21/60	24/60
Aspidospermine (cryst., Fraude)	1	6/10	4/10
	5	6/10	6/10

Table III

Effect of Parasympathetic Stimulants and Related Agents on Resistance to Anoxia

Agent	Dose (mg./kg.)	Mortality Ratios	
		Treated Mice	Control Mice
Acetylcholine iodide	20	5/10	4/10
Carbaminoylcholine chloride	5	3/10	5/10
Acetyl beta-methylcholine chloride	10	5/10	4/10
Neurine bromide	10	5/10	4/10
Physostigmine salicylate	1	2/18	9/20
	0.5	4/20	7/20
Prostigmine methylsulfate	0.5	15/15	1/20
	0.1	4/20	7/20
Pilocarpine hydrochloride	5	3/10	4/10
Arecoline hydrobromide	1	4/20	8/20
Betaine chloride	1,000	7/10	5/10

Table IV

Effect of Parasympathicolytics and Related Agents on Resistance to Anoxia

Agent	Dose (mg./kg.)	Mortality Ratios	
		Treated Mice	Control Mice
Atropine sulfate	4	11/30	5/30
	2	6/10	5/10
Hyoscyamine sulfate	2	10/30	5/30
Duboisine sulfate	2	6/10	5/10
Hyoscine hydrobromide	0.5	5/10	5/10
Homatropine hydrochloride	5	7/10	6/10
Eucatropine hydrochloride	10	8/10	6/10
Trasentin	15	5/10	6/10

Table V

Effects of Agents Acting on Autonomic Ganglia on Resistance to Anoxia

Agent	Dose (mg./kg.)	Mortality Ratios	
		Treated Mice	Control Mice
Nicotine (free base)	0.5	4/10	5/10
Lobeline sulfate	2	4/10	5/10
Sparteine sulfate	25	6/19	7/20

All agents were administered intraperitoneally. Those with prolonged effect were given thirty to sixty minutes before exposure of the mice to anoxia, while agents with brief duration of action were given within ten minutes of exposure. Lethal degrees of anoxia are not attained until some forty minutes after beginning exposure with the method used, however, so that results with short-acting agents must be interpreted cautiously.

RESULTS

Tables I to V present detailed results of treatment with different classes of agents affecting the autonomic nervous system, and Table VI is a summary of these data with the addition of results with cocaine which have been reported previously.[12] The mortality ratios signify the number of mice dying from anoxia divided by the total number of mice exposed. Mortality ratios for untreated control groups simultaneously exposed to anoxia are noted to the right of the corresponding treated groups. These bear no relation to mortality ratios of other control groups, since anoxia was terminated at different levels for different pairs

TABLE VI

SUMMARY OF EFFECTS OF GROUPS OF AGENTS ON RESISTANCE TO ANOXIA

GROUP	MORTALITY RATIOS		PER CENT MORTALITY	
	TREATED	CONTROLS	TREATED	CONTROLS
Sympathicomimetic agents (Table I)	342/500	168/500	68.4	33.6
Sympathetic sensitizer (cocaine)	13/20	11/20	65.0	55.0
Sympathicolytic agents (Table II)	52/150	76/150	34.7	50.7
Ergotamine, yohimbine, and 883F (Table II)	19/70	42/70	27.1	60.0
Parasympathicomimetic and related agents	57/153	58/160	37.3	36.3
Choline esters and neurine (Table III)	18/40	17/40	45.0	42.5
Other parasympathetic stimulants* (Table III)	17/88	35/90	19.3	38.9
Parasympathicolytics and trasentin (Table IV)	58/120	43/120	48.3	35.8
Ganglionic agents (Table V)	14/39	17/40	35.9	42.5

*Physostigmine, pilocarpine, arecoline, and prostigmine in a dose of 0.1 mg. per kilogram.

of treated and control groups and the resistance of mice to anoxia is not constant on different days.[12]

Following exposure to anoxia, the survivors were observed for twenty-four hours. No late effects of anoxia in treated mice were recognized.

Statistical treatment[13] of the combined data in Table VI demonstrates a significantly harmful effect of adrenergic and parasympathicolytic agents, and significant prophylactic effects of sympathicolytic agents and those with prolonged cholinergic effects. Short-acting cholinergic agents, those acting on autonomic ganglia, and the adrenergic sensitizer, cocaine, had no significant effects. The propriety of treating the combined data statistically may be questioned, but it is supported by the findings for individual agents. Significantly harmful effects were found for ephedrine, benzedrine, and its optical isomers, and propadrine, while significant prophylaxis was found with physostigmine, ergotamine, and the synthetic sympathicolytic, 883F. Other agents with similar actions would probably show significant effects if larger groups of mice were used.

DISCUSSION

A brief consideration of the properties of the individual agents is desirable, since none has a single, pure action on the autonomic nervous system. In several instances, lack of conformity with the general trend of the results is due to the short action of the agents in question. Otherwise, conclusions reached through consideration of results with groups of agents are generally supported for individual agents; that is, those with stronger adrenergic or parasympathicolytic activity tend to decrease resistance to anoxia more strongly, while cholinergic and sympathicolytic properties tend to prevent lethal effects of anoxia.

Adrenergic and related substances. Results with epinephrine and the isomers of benzedrine have been taken from previous reports[12, 14] in which their pertinent properties are adequately discussed. The sympathicomimetic action of epinephrine is the greatest of the agents studied, but this action is brief. Further, discharge of epinephrine from the adrenals at lethal levels of anoxia would make it unlikely that additional epinephrine or other sympathicomimetic agents would have desirable effects. Despite this improbability, ephedrine,

norephedrine, pseudonorephedrine, and benzedrine are claimed[9, 10] to delay respiratory failure in dogs subjected to fulminating anoxia. None has prophylactic value under the present experimental conditions. Synephrine, which is strongly adrenergic, is considered unsuitable[15] for the therapy of mountain sickness. These observations emphasize that agents which may be of temporary benefit in treating fulminating anoxia do not necessarily have any prophylactic value and may actually be harmful if used for prophylaxis. A study[12] of prophylactic effects of 12 respiratory stimulants confirms this. Conversely, agents such as alcohol with appreciable prophylactic activity are not necessarily suited for treatment of anoxia.[16]

Phenethylamine exerts mainly nicotine-like actions while phenethanolamine has a more purely adrenergic action.[17] Pseudoephedrine is weaker than ephedrine in most of its sympathicomimetic effects and is said to be a myocardial stimulant while ephedrine is depressant in full dose.[5] Hordenine causes vagal stimulation in small dose and paralyzes the vagus center in large dose but has definite adrenergic activity.[5] Tyramine may be considered as an adrenergic sensitizer similar to ephedrine; its effect in anoxia is not statistically significant. Norephedrine and pseudonorephedrine together constitute propadrine, of which the commercial preparation contains also a small amount of ammonium chloride. Despite the empirical use of ammonium chloride as a prophylactic against mountain sickness, propadrine is not less harmful than either of its fractions. Mescaline shares with benzedrine an enhanced activity on the central nervous system, but its effect in anoxia is not significant, although the stronger central stimulant isomer of benzedrine is significantly more harmful than the weaker isomer at one dose level.

Dihydroxyphenylalanine, "dopa," has no appreciable adrenergic properties but is related to the phenolic sympathicomimetic amines. Under the conditions of anoxia used, the redox functions of these agents are not of sufficiently long duration to act at lethal levels of anoxia. Octin, an aliphatic amine, has greater spasmolytic and less pressor effect than the true adrenergic agents, and its action in anoxia indicates that bronchodilatation is not an important factor in prophylaxis.

Sympathicolytic agents. The first three agents in Table II paralyze adrenergic functions for a relatively long period. The two dioxane derivatives act as mild narcotics in doses of 10 mg. per kilogram. Aspidospermine is a weak sympathicolytic and causes pulmonary edema and hyperemia.[8]

Cholinergic and related substances. The choline derivatives and neurine have a transient action. The remainder of the agents in Table III, except betaine, cause prolonged cholinergic effects. Physostigmine is most effective in anoxia in a dose within the lethal range, but prostigmine appears definitely harmful in high dose. Betaine is wholly inert as to cholinergic activity, but its chloride is strongly acid and is included as a control for the other salts used.

Belladonna group and trasentin. Although none of these agents has a significant harmful effect in anoxia, there is a close relationship between the parasympathicolytic potency and the detrimental effects in anoxia. Stimulant effects of these agents on respiration possibly mask the full effects of peripheral pa-

ralysis of the craniosacral nerves in anoxia. Hyoscine apparently does not act as a strong respiratory depressant in anoxia, and it is of interest that an antagonism of the respiratory effects of hyoscine and morphine has been noted clinically.[18] Properties of the five commonly used belladonna alkaloids need no further exposition. Duboisine is variously described as equivalent to hyoscyamine,[19] or to a mixture of hyoscyamine, hyoscine, and minor alkaloids.[20] Trasentin, or diphenylacetyldiethylaminoethanol hydrochloride, is not parasympathicolytic but is included as a control for the spasmolytic effects of the group. It may be noted that its effects in anoxia differ appreciably from those of octin.

Ganglionic agents. In addition to effects on autonomic ganglia, these agents have marked central effects, mediated in part through the carotid sinus mechanism. The resultant respiratory stimulation is brief. Thus, lobeline may be of some value in treatment of asphyxia neonatorum but is not prophylactic in anoxia. Sparteine affects autonomic ganglia but also potentiates epinephrine.[21] It acts as a myocardial poison and is included since it has been used clinically in conditions in which anoxia may occur; i.e., asthma and cardiac disease.

SUMMARY

Certain cholinergic and sympathicolytic agents have significant prophylactic actions against lethal effects of anoxic anoxia in mice, while adrenergic and parasympathicolytic agents tend to increase the lethal effects. Other classes of autonomic drugs are without appreciable effect. Differences in the therapeutic and prophylactic efficacies of individual agents are discussed.

REFERENCES

1. Van Liere, E. J.: Anoxia, Its Effect on the Body, Chicago, 1942, University of Chicago Press.
2. Monge, C. M., et al.: Fisiologia andina—circulation, An. de la Facultad de Ciencias médicas (Lima) 17: 1, 1935.
3. Ury, B., and Gellhorn, E.: Influence of Oxygen Deficiency on Reflex Dilatation of the Pupil, Proc. Soc. Exper. Biol. & Med. 38: 426, 1938.
4. Gasser, H. S., and Loevenhart, A. S.: The Mechanism of Stimulation of the Medullary Centers by Decreased Oxidation, J. Pharmacol. & Exper. Therap. 5: 239, 1914.
5. Gomez, L., and Pike, F. H.: The Histological Changes in Nerve Cells Due to Total Temporary Anaemia of the Central Nervous System, J. Exper. Med. 11: 257, 1909.
6. Sawyer, M. E. M., Schlossberg, T., and Bright, E. M.: Studies of Homeostasis in Normal and Sympathectomized Animals; Effect of Anoxemia, Am. J. Physiol. 104: 184, 1933.
7. Asher, L., and Streuli, H.: Beiträge zur Physiologie der Drüsen. XXXVI. Das Verhalten von schilddrüsenlosen, milzlosen, schilddrüsen- und milzlosen Tieren bei O_2-Mangel zugleich ein Beitrag zur Theorie der Bergkrankheit, Biochem. Ztschr. 87: 359, 1918.
8. Sollmann, T.: A Manual of Pharmacology, ed. 6, Philadelphia, 1942, W. B. Saunders Co.
9. Binet, L., and Strumza, M.: Les éphédrines agents actifs dans la lutte contra l'anoxémie, Compt. rend. Acad. d. sc. 207: 543, 1938.
10. Binet, L., and Strumza, M. V.: Le sulfate de benzédrine et la durée de résistance à l'anoxémie aiguë, Bull. et mém. Soc. méd. d. hôp. de Paris 55: 1264, 1939.
11. Emerson, G. A., and Van Liere, E. J.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. IV. A Standardized Technique, J. Lab. & Clin. Med. 28: 689, 1943.
12. Emerson, G. A., and Van Liere, E. J.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. I. Convulsant Agents, Arch. Internat. de pharmacodyn. et de thérap. 64: 239, 1940.
13. Loewenthal, L. J. A., and Wilson, W. A.: Problems of Chance in Medicine and Research, Brit. M. J. 2: 110, 1939.

14. Emerson, G. A.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. III. *d*-, *l*- and *dl*-Phenisopropylamines, J. Pharmacol. & Exper. Therap. 76: 11, 1942.
15. Anthony, A. J., and Atmer, S.: Luftfahrtmed. 1: 185, 1936; through Chem. Abstr. 31: 4394, 1937.
16. Emerson, G. A., Van Liere, E. J., and Morrison, J. L.: Drug Prophylaxis Against Lethal Effects of Severe Anoxia. II. Alcohol, Amytal and Pentobarbital, Proc. Soc. Exper. Biol. & Med. 49: 376, 1942.
17. Alles, G. A., and Knoefel, P. K.: Comparative Physiological Actions of Phenethylamine and of the Betahydroxyphenethylamines, Univ. California Publ., Pharmacol. 1: 101, 1938.
18. Waters, R. M., Bennett, J. H., and Leigh, M. D.: Effects Upon Human Subjects of Morphine and Scopolamine Alone and Combined, J. Pharmacol. & Exper. Therap. (Proc.) 63: 38, 1938.
19. Merck's Index, ed. 4, Rahway, N. J., 1930.
20. The Merck Index, ed. 5, Rahway, N. J., 1940.
21. Koppanyi, T., and Linegar, C. R.: The Effect of Sparteine on the Autonomic Nervous System, J. Pharmacol. & Exper. Therap. (Proc.) 72: 23, 1941.

COMPARATIVE TOXICITY OF PENTOBARBITAL IN THE NEWBORN AND ADULT RAT*

B. Etsten, F. A. D. Alexander, and H. E. Himwich
Albany, N. Y.

Recent studies have disclosed that newborn rats, dogs, cats, and rabbits exhibit an extraordinary tolerance to anoxia[1] produced by the inhalation of pure nitrogen, helium, or carbon dioxide, or by the volatile narcotics, nitrous oxide and cyclopropane. The adult rat usually dies within a period of three minutes when respiring either pure nitrogen or these depressant drugs, whereas the newborn of the same species survives for approximately fifty minutes in nitrogen and nitrous oxide, and twenty-five minutes in cyclopropane and carbon dioxide. The newborn rats are also relatively resistant to alcohol[2] and to the acute lethal effects of morphine.[3] On the other hand, the newborn appears relatively more sensitive than the adult to the nonvolatile depressant drug, pentobarbital. Rosenfeld and Snyder,[4] and Windle and Becker[5] have shown that the barbiturates depress the respiratory efforts of the fetus more than those of the pregnant mother. In view of the discrepancy between the tolerance of the newborn to anoxia and the volatile anesthetics and the susceptibility of the fetus to barbiturates, the present comparative studies of the susceptibility and resistance of the newborn and adult rat to pentobarbital sodium were made. These observations include fetuses and pregnant rats near term.

In an effort to determine the mechanism of the action of barbiturates, the effect of pentobarbital on the survival time of the newborn in nitrogen and nitrous oxide was examined.

METHOD

Rats 1 to 2 days old and mature animals were injected intraperitoneally with various concentrations of pentobarbital. The tolerance of the newborn was

*From the Departments of Anesthesia and Physiology and Pharmacology, Albany Medical College, Union University, Albany, N. Y.

Aided by a grant from the Albion O. Bernstein Memorial Fund.
Received for publication, August 3, 1942.

compared with that of the adult animal given an equivalent concentration of barbiturate. In this manner the lethal dose for the newborn and the adult was determined. Cessation of respiration and inability to evoke respiration were taken as the criteria of the lethal effect.

Pregnant rats were subjected to various concentrations of pentobarbital. After allowing sufficient time for the absorption of the drug, cesarean section was done in order to study the effect on the fetus. One rat already in labor was injected with an anesthetic dose of pentobarbital, and the fetuses were observed after expulsion from the uterus. Infant rats subjected either to anesthetic or lethal doses of pentobarbital were placed in an atmosphere of pure nitrogen. The survival time of these newborn rats was compared with that of the uninjected litter mate controls. Other infant rats who had received lethal doses of pentobarbital were injected with various concentrations of metrazol to determine the combined effects of both drugs on respiratory depression and survival period. A control study was made to ascertain the tolerance of the newborn for metrazol.

TABLE I

SURVIVAL PERIOD OF NEWBORN AND ADULT RATS INJECTED WITH PENTOBARBITAL (0.1%)

NO. OF ANIMALS	AGE GROUP	PENTOBARBITAL (MG./GM.)	RESULTS	AVERAGE SURVIVAL TIME
3	Newborn	0.8	3 died	30 minutes
4	Newborn	0.5	4 died	35 minutes
4	Newborn	0.4	4 died	45 minutes
20	Newborn	0.3	16 died	60 minutes
8	Newborn	0.2	8 lived	Anesthetic dose
1	Adult	0.2	1 lived	No anesthesia
3	Adult	0.3	3 lived	No anesthesia
3	Adult	0.4	3 lived	Surgical anesthesia
6	Adult	0.5	6 lived	Surgical anesthesia
1	Adult	0.6	1 died	32 minutes
1	Adult	0.7	1 died	30 minutes

RESULTS

Table I reveals that 0.3 mg. per gram of body weight of pentobarbital is the minimal fatal dose for the rat 1 to 2 days old. Sixteen of 20 newborn animals died after an average survival period of sixty minutes, with a range of thirty to ninety minutes. All of 8 newborn animals receiving 0.2 mg. per gram survived, whereas 4 animals receiving 0.4 mg. per gram all died within forty-five minutes. The minimal fatal dose for adult rats of our colony is 0.5 mg. per gram of body weight. Each of 6 adult rats receiving this dose died after an average survival of thirty minutes. Adult rats receiving 0.3 mg. per gram were not even anesthetized. Those receiving 0.4 mg. per gram exhibited a short period of surgical anesthesia. In Table II is disclosed the sensitivity of fetal rats to pentobarbital. Three pregnant rats received pentobarbital in doses ranging from 0.3 mg. per gram to 0.4 and 0.5 mg. per gram of body weight. Twenty-nine fetuses were born spontaneously or were delivered by cesarean section fourteen minutes after the injection of the drug. All fetuses died from ten to forty minutes after the delivery, and the extrauterine survival time decreased with increasing dosage of pentobarbital. In contrast all the mothers with the exception of one which received 0.5 mg. per gram survived.

TABLE II

EFFECTS OF PENTOBARBITAL (0.1%) ON FETAL RATS

NO. OF PREGNANT RATS	PENTOBARBITAL (MG./GM.)	PROCEDURE	NO. OF FETUSES	RESULTS	SURVIVAL PERIOD OF FETUSES
1	0.3	Cesarean section	9	9 died	40 minutes
1	0.4	Cesarean section	10	10 died	10 minutes
1	0.5	Section after death	10	10 died	10 minutes
1	0.3	Spontaneous delivery	4	3 died	45 minutes

TABLE III

EFFECTS OF PENTOBARBITAL (0.1% AND 0.3%) AND ANOXIA ON SURVIVAL PERIOD OF NEWBORN RATS

NO. OF NEWBORN RATS	PENTOBARBITAL (MG./GM.)	AGENT	RESULTS	AVERAGE SURVIVAL TIME
3	0.1	Nitrogen	3 died	50 minutes
3	0.1	Nitrous oxide	3 died	50 minutes
2	0.1	Nitrogen	2 lived	40 minutes
6	0.3	Nitrogen	6 died	25 minutes

A rat which had delivered 4 of her fetuses was injected with 0.3 mg. per gram of pentobarbital. Eighteen minutes after the injection one fetus was born spontaneously. It exhibited no signs of respiratory depression and survived. Three fetuses born from forty-five to ninety minutes after the injection were in apnea. Respirations could not be evoked and all 3 died. Table III presents the data on infant rats placed in an atmosphere of nitrogen or nitrous oxide after receiving 0.1 mg. per gram, an anesthetic dose, or 0.3 mg. per gram, a lethal dose, of pentobarbital. Six newborn rats receiving the lower dose survived for fifty minutes either in nitrogen or nitrous oxide. However, the infant rats with the larger dose survived on an average only twenty-five minutes.

TABLE IV

EFFECTS OF METRAZOL (10 PER CENT) ON SURVIVAL PERIOD OF NEWBORN RATS

NO. OF NEWBORN RATS	METRAZOL (MG./GM.)	EFFECT	RESULT
6	5.0	Convulsion	6 died
5	4.0	Convulsion	5 died
5	3.0	Slight convulsion	5 lived
5	2.0	Slight convulsion	5 lived

Table IV shows the results of the experiments made to determine the lethal dose of metrazol for newborn rats. Five animals injected with 3.0 mg. per gram of body weight exhibited mild convulsive movements and survived. With larger doses 4.0 and 5.0 mg. per gram the animals died as a result of the severe convulsions.

TABLE V

EFFECT OF PENTOBARBITAL (0.3 MG./GM. BODY WEIGHT) AND METRAZOL (10%) ON SURVIVAL PERIOD OF NEWBORN RATS

NO. OF NEWBORN RATS	METRAZOL (MG./GM.)	INJECTION TIME AFTER PENTOBARBITAL	RESULT
5	7.5	20 minutes	5 lived
5	5.0	15 minutes	5 lived
5	4.0	22 minutes	5 lived
5	3.0	35 minutes	5 lived

Table V presents the effects of various concentrations of metrazol on infant rats given a lethal dose of pentobarbital. It may be seen that 3.0, 4.0, and 5.0 mg. per gram of metrazol successfully antagonize lethal doses of pentobarbital.

DISCUSSION

In contrast with the extraordinary tolerance of the newborn rat to the volatile anesthetics, nitrous oxide and cyclopropane, the infant rat both ante partum and post partum is more sensitive to pentobarbital than is its mother. The newborn rat died with a dose of 0.3 mg. per gram and the adult with 0.5 mg. per gram. Pentobarbital depresses the respiratory centers so that the animal cannot be resuscitated and dies from anoxia. Since the newborn rat is more tolerant of anoxia than is the adult, the greater sensitivity of the infant rat to barbiturate cannot be explained simply by the anoxia produced as a result of the depression of respiration. The mechanism which normally facilitates the survival of the newborn rat during anoxia depends upon the anaerobic splitting of carbohydrate with the formation of lactic acid.[6, 7] It is true that nonfatal doses of barbiturate do not shorten the survival period of the newborn rats in an atmosphere of nitrogen. However, a fatal dose of pentobarbital (0.3 mg. per gram) which permits the infant rat to survive for sixty minutes in air cuts down the survival period in nitrogen to twenty-five minutes. This is half the period in which the infant rat can survive in pure nitrogen. It would, therefore, seem that pentobarbital inhibits the mechanism for the anaerobic production of energy from carbohydrate. One path of the breakdown of carbohydrate involves a series of the same intermediates from glucose to the formation of pyruvic acid irrespective of the presence or absence of oxygen. Pyruvic acid, however, is then either oxidized to carbon dioxide and water, or in the absence of oxygen is converted to lactic acid. It would, therefore, seem that pentobarbital interferes with some stage in glucose metabolism before the formation of pyruvic acid. In any case lactic acid formation is not increased by the barbiturates.[8] Quastel and Wheatley[9] have previously indicated that barbiturates depress aerobic sources of cerebral energy. The present evidence reveals that barbiturates probably also inhibit the anaerobic breakdown of carbohydrate.

Though the newborn rat is susceptible to pentobarbital, it is able to withstand a lethal dose with the aid of metrazol. The mutual antagonism between the two drugs is emphasized because the dose of metrazol used would have been lethal for an animal that had not received pentobarbital.

CONCLUSIONS

1. The newborn rat is more susceptible than the adult to pentobarbital both ante partum and post partum. The lethal dose for the newborn rat is 0.3 mg. per gram, and for the adult 0.5 mg. per gram.

2. Evidence is afforded indicating that pentobarbital inhibits the development of the anaerobic energy required for survival in the absence of oxygen.

3. Doses of pentobarbital and metrazol, which when given singly are lethal, may successfully antagonize each other and facilitate survival when administered together.

REFERENCES

1. Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E.: Tolerance of the Newborn to Anoxia, Am. J. Physiol. 131: 281, 1941.
2. Chesler, A., LaBelle, G. C., and Himwich, H. E.: The Relative Effects of Toxic Doses of Alcohol on Fetal, Newborn and Adult Rats, Quart. J. Alcohol 3: 1, 1942.
3. Chesler, A., LaBelle, G. C., and Himwich, H. E.: A Study of the Toxic Effects of Morphine on the Fetal, Newborn and Adult Rats, J. Pharmacol. & Exper. Therap. 75: 363, 1942.
4. Rosenfeld, M., and Snyder, F. F.: The Factor of Anesthesia in the Pathogenesis of Asphyxia Neonatorum, Am. J. Obst. & Gynec. 38: 424, 1939.
5. Windle, W. F., and Becker, R. F.: Role of Carbon Dioxide in Resuscitation at Birth After Asphyxia and After Nembutal Anesthesia, Am. J. Physiol. 133: 493, 1941.
6. Himwich, H. E., Bernstein, A. O., Herrlich, H., Chesler, A., and Fazekas, J. F.: Mechanisms for the Maintenance of Life in the Newborn During Anoxia, Am. J. Physiol. 135: 387, 1942.
7. Himwich, H. E., Herrlich, H., Rich, E., Barstow, R., and Fazekas, J. F.: Effect of Iodoacetate on Survival Period During Hypoxia, Federation Proc. 1: 40, 1942.
8. Rakieten, N., Nahum, L. H., DuBois, D. Gildea, E. F., and Himwich, H. E.: The Effect of Some Compounds of Barbituric Acid and of Urethrane, J. Pharmacol. & Exper. Therap. 50; 328, 1934.
9. Quastel, J. H., and Wheatley, A. H. M.: Narcosis and Oxidations of Brain, Proc. Roy. Soc., London, S. B. 112: 60, 1932.

SPECIES OF THE TRIBES MIMEAE, NEISSERIEAE, AND STREPTOCOCCEAE WHICH CONFUSE THE DIAGNOSIS OF GONORRHEA BY SMEARS*

George G. De Bord, Ph.D., Washington, D. C.

FORTY years ago gram-negative diplococci from spinal fluids[1] or conjunctivitis[2] were considered meningococci. Such statements were refuted[3] at the time and since then it has been proved quite conclusively by other investigators[4-6] that diplococci other than the meningococci can cause a meningitis. Also during that time gram-negative diplococci in smears from conjunctivitis and vaginitis have been considered gonococci, although gram-positive diplococci which lose the Gram stain,[7, 8] lactose-fermenting rods which appear microscopically to be gram-negative diplococci,[9] and a species of the genus Neisseria other than the gonococcus in a rape case[10] have been reported. The investigations of Rossiter[11] on Samoan conjunctivitis were probably the first which proved definitely that a gram-negative diplococcus indistinguishable from the gonococcus in smear preparations was present in one type of conjunctivitis. This conjunctivitis had been diagnosed previously by smear preparations as gonorrheal despite the fact that venereal gonorrhea was not known on the island at that time.

The present investigation was undertaken to study the flora of conjunctivitis and vaginitis with special reference to the gram-negative diplococci. The cases studied included the newborn; children with conjunctivitis; girls of prepuberty age, both normal and those with varying degrees of vaginitis; and one case of conjunctivitis in an adult male.

*From the District of Columbia Department of Health.
Received for publication, August 5, 1942.

EXPERIMENTAL

The medium used for isolation was a chocolate agar made from Difco proteose peptone No. 3 with 5 per cent citrated human blood. Later rabbit blood was substituted for human blood. Concentrated sugar solutions in distilled water were sterilized at 15 pounds pressure for fifteen minutes and added aseptically so that the final dilution in broth was 1 per cent. Acetone was used as the decolorant in the Gram stain. All media which were not used as they came from the autoclave were incubated for twenty-four hours before use. A sterile cotton swab was used to obtain the specimen which was streaked immediately over the plate. Each type of colony, with some duplications, was examined microscopically for gram-negative diplococci. The examinations for Neisseriae were made by the oxidase test with dimethyl-p-phenylenediamine. Many species, especially gram-negative rods, give this test, which makes it essential to examine the colonies microscopically and culturally for the correct determination of the species if gram-negative diplococci be present. This confirms the work of Gordon and McLeod[12] and Price.[13]

One hundred and forty-seven cases were studied. These included 41 patients with purulent conjunctivitis but did not include many other cases in which the eyes showed a mild discharge with a few intracellular or extracellular gram-negative diplococci in the direct smears and no species of Neisseria on the plates. Of the 41, 10 were infections with *Neisseria gonorrhoeae,* two cases showed *Neisseria catarrhalis,* and one showed *Neisseria fulva.*[14] The one adult male case showed *Neisseria intracellularis.* This species was shown to be serotype I when typed by Dr. Sara Branham of the U. S. Public Health Service. Twelve cases showed species of the tribe Mimeae,[14] of which two were *Herellea vaginicola* and *Colloides anoxydana.* Species of the remaining cases were not classified except to show that Neisseriae were not present.

Forty-nine cases showed vaginitis. Five were infections with *N. gonorrhoeae. N. fulva* was isolated once, three cases showed *N. catarrhalis,* and nine cases showed species of the tribe Mimeae, two of which were *Mima polymorpha* var. *oxidans* and one was *C. anoxydana.* The species of the remaining cases were not investigated further than to show that no species of the genus Neisseria was present.

In the group of 57 clinically normal cases, many showed an occasional intracellular or extracellular gram-negative diplococcus in the smear. One case showed *Neisseria gigantea,*[14] two showed *N. catarrhalis,* and 24 cases showed Mimeae of which two were *Mima polymorpha,* two were *M. polymorpha* var. *oxidans,* and one each of the species *H. vaginicola* and *C. anoxydana.*

DISCUSSION

The presence of gram-negative diplococci which were not Neisseriae was found by a systematic search. This was done by making a stained smear of every type, with some duplications, of colonies on the plate. Those species which appeared to be gram-negative diplococci were then studied culturally. The visual examination of the colonies is not sufficient because it is not possible to differentiate Mimeae, many forms of the gram-positive diplococci, and many forms of the diphtheroids by their colonial characters on plates. The three tribes,

Neisserieae, Streptococceae, and Mimeae, represented by the genera Neisseria, Mima, Herellea, Colloides, and the gram-positive diplococci which tend to lose the stain were found to be present in conjunctivitis and vaginitis. All these species are either true gram-negative diplococci or have forms which are gram-negative diplococci.

The presence of species of the genus Neisseria other than *N. gonorrhoeae* must be considered when diagnoses are made upon the basis of morphology. While these species are relatively less important when judged by the number which have been found in conjunctivitis and vaginitis, the possibility of their presence is one factor which must be considered when evaluating the reliability of smear examinations. The presence of *N. intracellularis* in the one case could have been detected only by cultural methods, thereby enhancing those methods in conjunctivitis. *N. intracellularis* and *N. fulva* were present in practically pure culture in both cases in which they occurred. The presence of *N. gigantea* could explain the statements in the literature that some cultures of the gonococcus showed large forms. The inability of *N. gigantea* to ferment sugars would not permit its presence to be recognized as a contaminant of cultures of *N. gonorrhoeae* because the fermentative reactions of *N. gonorrhoeae* would not be changed. Only the microscope would reveal the larger form. *N. catarrhalis* and *N. gigantea* were present in relatively few numbers in the cases in which they were found.

A second group of minor importance is the gram-positive diplococci which tend to lose the Gram stain. Schubert and Toenges[7] included certain streptococci in this group also. These species may be present in either vaginitis or conjunctivitis. The number of cases involved is low, and a systematic search by staining smears from many individual colonies on the plate is necessary to find those which decolorize, although they do predominate occasionally. The gram-positive diplococci which tend to lose the stain cannot be differentiated on the plates from those which do not decolorize because their colonial characters are the same and those which do not decolorize may be present in large numbers in vaginitis and conjunctivitis. The pairs of cocci which have decolorized are identical in appearance to the gonococcus when the size is the same.

The most important group is the tribe Mimeae. The prevalence of this tribe complicates the microscopic picture to such an extent that it is imperative to use cultural methods to obtain a true evaluation of the flora.

Since the microscopic picture of the species of the tribe Mimeae is identical to the genus Neisseria, except *N. gigantea,* all species of the tribe interfere in the diagnosis of gonorrhea by smears and *M. polymorpha* var. *oxidans* can interfere in the cultural method when tests are made presumptively by the oxidase test for the presence of Neisseriae.

The question of pathogenicity with these species cannot be answered categorically because Koch's postulates have not been fulfilled with the original animal, the human being. There are, however, several points indicating that they may be primary or secondary invaders. First, the diplococcal form is the more prominent at the primary isolation and the diplococcus/rod ratio[14] falls upon artificial cultivation. Second, extensive animal inoculations have not been done, but species having low diplococcus/rod ratios injected intraperitoneally in rela-

tively large numbers have been recovered with high ratios from the heart blood of the mice after death. Third, in conjunctivitis in the newborn where the data can be obtained, the infection arises very consistently from the fifth to the seventh day after birth and has persisted for as long as two months. Fourth, a change from an unbuffered silver nitrate solution to a buffered one used in the eyes at birth reduced the number of cases materially. Fifth, with all other supporting evidence lacking, pathogenicity might be assumed when only one species is present in the infection. This condition has not always been true, but in the cases with *N. intracellularis, N. fulva,* and some of the Mimeae in conjunctivitis, also with *N. fulva* and one case with *M. polymorpha* var. *oxidans* in vaginitis, the number of colonies of other species on the plate was negligible. It was noted that the species of Mimeae with greater fermentative powers, some of which have not been described, were more prevalent in conjunctivitis and vaginitis than those of the genus Mima which does not ferment carbohydrates. Proof is lacking whether this might be due to a greater pathogenicity or merely accidental circumstances. Whether any of these species are pathogenic or not, they constitute important sources of error which confuse the diagnosis of gonorrhea by smears.

As a result of the probable presence of one or more of the three groups, Mimeae, Neisserieae, gram-positive diplococci which tend to lose the stain, it is impossible to make logical comparisons between direct smears and cultures in conjunctivitis and vaginitis. In the smears the gram-negative diplococci of the three groups are not differentiated from the gonococcus, rods and filaments cannot be used as a basis for determining the presence of the pleomorphic Mimeae, and gram-positive diplococci would not prove whether it was a species that tended to lose or retain the stain. There is, therefore, no criterion which would indicate the presence of these species in stained preparations, and it would be entirely possible for a diagnosis of gonorrhea to be given from a smear preparation in conjunctivitis or vaginitis, even though a pure culture of either Mimeae or gram-positive diplococci which tend to lose the stain was present. The Mimeae, with the exception of *M. polymorpha* var. *oxidans,* and the gram-positive diplococci which tend to lose the stain would not be detected by the oxidase test on the plate, and their presence could be determined only by a systematic search for gram-negative diplococci.

The statement that the gonococcus is difficult to grow may be explained by the presence of one or more of the tribes, Mimeae, Neisserieae, Streptococceae, which show as gram-negative diplococci in stained preparations but could not be detected readily on the cultural plate unless a systematic search were instituted for them. Neither the presence of gram-negative diplococci in a stained smear nor the oxidase reaction on a plate proves the presence of *N. gonorrhoeae.*

SUMMARY AND CONCLUSIONS

From the 147 cases of conjunctivitis, vaginitis, and normal vaginas, Mimeae were isolated forty-five times; gram-positive diplococci, which tend to lose the stain, six times; and Neisseriae, excluding the gonococcus, eight times. The total, 59, represents about 40 per cent in which real or apparent gram-negative diplococci were present which can be mistaken easily for *Neisseria gonorrhoeae.*

As a result of this investigation it is felt that the identification of *N. gonorrhoeae* by smears in conjunctivitis or vaginitis is not justified.

Appreciation is expressed to Dr. George C. Ruhland, Health Officer of the District of Columbia, who has made this investigation possible by his cooperation and interest. Acknowledgments are due Dr. H. H. Leifer for his many courtesies and collaboration.

REFERENCES

1. Jaeger, H.: Zur Aetiologie der Meningitis cerebrospinalis epidemica, Ztschr. f. Hyg. 19: 351, 1895.
2. Frankel, C.: Ueber das Vorkommen des Meningococcus intracellularis bei eitrigen Entzündungen des Augenbindehaut, Ztschr. f. Hyg. 31: 221, 1899.
3. Albrecht, H., and Ghon, A.: Zur Frage der morphologischen und biologischen Charakterisierung des Meningococcus intracellularis, Centralbl. f. Bakt., 1 Abt., Orig. 33: 496, 1902-03.
4. Moersch, F. P., and Thompson, L.: Meningitis Due to Microcatarrhalis, Journal-Lancet 48: 407, 1928.
5. Benson, H., Brennwasser, R., and D'Andrea, D.: Neisseria subflava (Bergey) Meningitis in an Infant, J. Infect. Dis. 43: 516, 1928.
6. Branham, S. E.: A New Meningococcus-like Organism (Neisseria flavescens n. sp.) From Epidemic Meningitis, Pub. Health Rep. 45: 845, 1930.
7. Schubert, M., and Toenges, T.: Mikroskopische und Kulturelle Untersuchungen über gram negative, den Gonokokken ähnliche Diplokokken im weiblichen Urogenitaltrakt, Dermat. Ztschr. 62: 362, 1931.
8. Skomorovskaia, A.-G.: Sur l'importance des formes involutines du staphlocoque pour le diagnostic du gonocoque, Ann. d. mal. vén. 32: 188, 1937.
9. Barbellion, P.: Colibacille simulant le gonocoque, J. de méd. de Paris 57: 76, 1937.
10. Cohn, A.: The Importance of Bacteriologic Cultures for the Diagnosis of Gonococcal Vulvovaginitis and Proctitis in Children, Am. J. Syph., Gonor. & Ven. Dis. 20: 623, 1936.
11. Rossiter, P. S., Supplementary Report of the Investigations of Samoan Conjunctivitis, U. S. Nav. M. Bull. 3: 349, 1909.
12. Gordon, J., and McLeod, J. W.: The Practical Application of the Direct Oxidase Reaction in Bacteriology, J. Path. & Bact. 31: 185, 1928.
13. Price, I. N. O.: The Oxidase Reaction in the Laboratory Diagnosis of Gonorrhea, Brit. M. J. 1: 199, 1929.
14. De Bord, G. G.: Descriptions of Mimeae trib. nov. With Three Genera and Three Species and Two New Species of Nesseria From Conjunctivitis and Vaginitis, Iowa State College J. Sc. 16: 471, 1942.

EFFECT OF ETHYL ALCOHOL INTOXICATION ON THE DEVELOPMENT OF LOCAL INFLAMMATORY REACTIONS IN THE RABBIT

R. H. Rigdon, M.D., Memphis, Tenn.

Pickrell[1] in 1938 reviewed the literature on the effect of intoxication on infection and immunity. He states that "while it is rather generally agreed that infected intoxicated animals die sooner than similarly infected, nonintoxicated ones, it is fair to say that very few of the studies have been adequately controlled. . . . No satisfactory explanation has as yet been established for any resistance lowering effect that alcoholic intoxication may exert."

From an experimental study made upon a large series of rabbits, Pickrell[1] concludes that "alcoholic intoxication maintained at the point of stupor, de-

*From the University of Tennessee, Department of Pathology, Memphis.

Aided by grants from the John and Mary R. Markle Foundation and the University of Tennessee.

Received for publication, August 12, 1942.

stroys resistance to pneumococcal infection. . . . The loss of resistance to infection appears to be due to the fact that intoxication profoundly inhibits the vascular inflammatory response. . . . In the absence of capillary dilatation and margination of the leucocytes, leucocytic emigration at the site of infection is negligible. . . . In what manner intoxication acts to prevent the usual inflammatory alterations in the capillaries is at present obscure but that it does so is clearly evident.''

Cressman and Rigdon[2] studied capillary permeability and inflammation in narcotized rabbits and found that ''capillary permeability in areas of inflammation is altered in rabbits narcotized with alcohol or ether, as demonstrated by the localization and concentration of trypan blue. . . . There is also a marked diminution in the number of leucocytes in the areas of inflammation in the skin in the narcotized rabbits.'' The observations of Cressman and Rigdon, therefore, are similar to those of Pickrell; however, neither have explained adequately the mechanism of this phenomenon.

Rigdon and associates have recently demonstrated that capillary permeability and inflammation in the skin of the rabbit may be affected by changes in the blood pressure; that is, when the pressure is abnormally low, the macroscopic and microscopic changes characteristic of inflammation either fail to appear or they are markedly decreased in the skin. It has been suggested that the peripheral vessels in the skin become constricted following the loss of blood,[3] sectioning of the cervical portion of the spinal cord,[4] and in staphylococcus septicemia in the rabbit.[5] The blood apparently does not circulate in the small blood vessels in the skin of these animals since the skin becomes pale, cold, and clammy. This absence of a normal circulation apparently may account for the absence of a leucocytic reaction about various irritants when they are injected intradermally into the animals.

Alcohol in large amounts will produce a lowering of the blood pressure.[6] This change in the pressure apparently may result from the effect of the alcohol on the heart. In view of our present knowledge of the effect of lowered blood pressure on the development of an inflammatory reaction in the skin of the rabbit, it seems important to restudy the effect of ethyl alcohol intoxication on the development of the inflammatory reaction in the skin of the rabbit. Observations are also made on the development of the inflammatory process in the rabbits' intestines since the circulation here apparently remains intact while that in the skin may become either markedly decreased or even absent in severe intoxication.

METHODS AND MATERIALS

Adult rabbits, varying from 1.5 to 2.5 kg. are used. The skin over the abdomen is carefully shaven twenty-four hours or longer before the experiments are begun. A 20 per cent dilution of ethyl alcohol is given through a small catheter into the stomach. Each rabbit is given 80 c.c. of alcohol, and smaller quantities are given subsequently if necessary to obtain complete anesthesia. The blood pressure is recorded at intervals from the carotid artery.

Four-tenths cubic centimeter of a twenty-four-hour broth culture of staphylococci are injected intradermally usually six, four, and two hours before the rabbits are killed. An incision is made in the abdominal wall; the intestines are exposed, and 0.4 c.c. of the broth culture is injected into the wall of the colon

of some of these rabbits. The animals are killed from two to six hours following the injection of the staphylococci. The areas of tissue injected with the cultures are removed and fixed immediately in a 10 per cent solution of formalin. Paraffin sections are prepared and they are stained with hematoxylin and eosin.

To study the effect of alcohol intoxication on capillary permeability, the abdomen of a group of rabbits is exposed to infrared radiation.* The light is placed 10 inches away from the skin. Immediately following the application of the light, 10 c.c. of a 0.2 per cent solution of trypan blue are given intravenously. This dye localizes and concentrates in the skin around the margin of the irradiated areas in normal rabbits and also in the coils of intestines that lie immediately beneath the portion of abdominal wall that is irradiated. The blood pressure is recorded from the carotids in these animals.

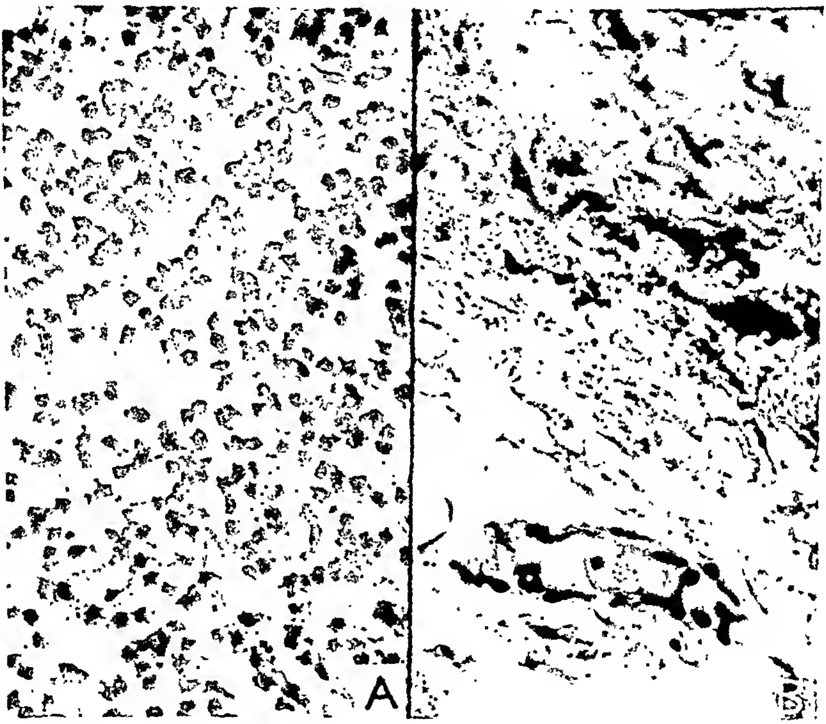


Fig. 1.—*A*, Rabbit 1192. Normal rabbit, 0.4 c.c. of twenty-four-hour broth culture injected intradermally six hours before section removed. There is a diffuse infiltration of polymorphonuclear leucocytes in the corium. *B*, Rabbit 1213. Rabbit given 70 c.c. of 20 per cent alcohol at 8:40 A.M.; blood pressure 80 at 9:50 A.M.; 0.4 c.c. broth culture of staphylococci injected intradermally at 10:20 A.M.; blood pressure 60 at 1:00 P.M., and 40 at [illegible] d at 4:30 P.M. The corium is infiltrated with bacteria; but there are n [illegible] the area.

As a control for this experiment on capillary permeability one group of rabbits is given pentobarbital intravenously; a second group is unanesthetized except for using 1.0 c.c. of a 1.0 per cent solution of novocaine in the neck before putting the cannula into the carotid artery. Infrared irradiation and trypan blue are given in a manner similar to that in the experimental group. The presence or the absence of trypan blue in the skin is recorded. The rabbits are killed at intervals varying from thirty minutes to four hours following the injection of the dye.

*The infrared lamp is supplied by the Burdick Company of Milton, Wisconsin. It is type Z-70, Zoalite.

LOCALIZATION OF POLYMORPHONUCLEAR LEUCOCYTES ABOUT STAPHYLOCOCCI INJECTED INTRADERMALLY IN NORMAL AND INTOXICATED RABBITS

Five normal rabbits are injected intradermally with the broth culture of staphylococci. Edema and hyperemia develop around the site of the injection. Polymorphonuclear leucocytes infiltrate the tissues within a period of two hours. The number of leucocytes rapidly increase during the subsequent four hours (Fig. 1*A*).

Sixteen rabbits are given alcohol. When completely anesthetized, a cannula is put into the carotid artery and the pressure is recorded. It is usually decreased at this time from the normal of 100-120 mm. Hg. The pressure may continue to fall or it may be necessary to give a second dose of alcohol in order to lower the pressure. The bacteria are not injected until the pressure reaches a level of approximately 70 mm. Hg or even less. The macroscopic reaction in the skin apparently is proportional to the level of the blood pressure. When the pressure is approximately 60 mm. Hg, the edema and hyperemia are markedly reduced. There is essentially no macroscopic reaction when the pressure is either 50 mm. or less. Histologically the number of polymorphonuclear leucocytes are proportional to the level of the blood pressure. When the pressure is either 50 mm. Hg or less, there are essentially no polymorphonuclear leucocytes about the clumps of bacteria (Fig. 1*B*).

LOCALIZATION OF POLYMORPHONUCLEAR LEUCOCYTES ABOUT STAPHYLOCOCCI INJECTED INTO THE WALL OF THE COLON OF RABBITS INTOXICATED WITH ETHYL ALCOHOL

In the previous experiments it was shown that polymorphonuclear leucocytes fail to localize and to concentrate about groups of staphylococci in the skin of rabbits intoxicated with ethyl alcohol when the blood pressure is abnormally low. A group of six rabbits are given a quantity of alcohol equal to that given to the animals in the preceding experiments. Staphylococci are injected into the wall of the colon and into the skin of these rabbits. The blood pressure is recorded during a period of four to six hours subsequently. When the pressure is abnormally low, there are no macroscopic evidences of inflammation in the skin. There are macroscopic evidences of inflammation, however, in the intestines about the point of injection of the bacteria. Histologically, there are no polymorphonuclear leucocytes about the bacteria in the skin, although many leucocytes have infiltrated the wall of the intestines about the point of injection of the bacteria (Fig. 2).

The results of this experiment indicate that intoxication per se is not the direct cause of the failure of leucocytes to localize and to concentrate about collections of staphylococci. If the bacteria are in the wall of the colon, the leucocytes localize and concentrate in a manner similar to that in an unintoxicated animal. Furthermore, the results suggest that the reaction in the skin is directly affected by the level of the blood pressure. When the pressure is abnormally low, the skin becomes pale, cold, and clammy, indicating a disturbed circulation in the peripheral vessels. The blood flow through the intestine of the same rabbits apparently is not similarly decreased. Polymorphonuclear leucocytes continue to localize and to concentrate about staphylococci as they do in an unintoxicated rabbit.

OBSERVATIONS ON CAPILLARY PERMEABILITY IN THE SKIN AND INTESTINES OF INTOXICATED RABBITS

In unpublished experiments it has been found that infrared radiation when applied to the skin of a normal rabbit for five minutes produces both macroscopic and microscopic evidences of inflammation. Similar pathologic changes also occur in the wall of the intestines. Trypan blue injected intravenously into these rabbits localizes and concentrates in both the treated areas of skin and in the intestines.

Fig. 2.—Rabbit 1213. Same rabbit as shown in Fig. 1 *B*. 0.4 c.c. of the broth culture of staphylococci was injected into the wall of the colon six hours before the rabbit was killed. There are many leucocytes infiltrating the area.

Fig. 3.—Rabbit 1294. Rabbit given 80 c.c. of alcohol at 9:30 A.M. Blood pressure 80 at 10:15, 60 at 10:30, 35 at 11:05, and 30 at 11:10. Infrared light is applied for five minutes to skin of abdomen in area outlined with India ink at 11:20; 10 c.c. of trypan blue are given intravenously immediately following the light. At 12:00 there is no trypan blue in the skin, but the portion of intestines immediately beneath the treated area of skin is dark blue in color.

In the study of the effect of alcohol intoxication on capillary permeability six rabbits are given alcohol. The blood pressure is recorded. When it is abnormally low (approximately 50 mm. Hg), the infrared lamp is placed over the abdomen for a period of five minutes. The macroscopic reaction in the skin is insignificant. The animals are given trypan blue intravenously immediately following the irradiation. A minute amount of the dye localizes in the skin of some of the rabbits; however, no dye localizes in the skin of the majority of the animals (Fig. 3). The coils of intestines beneath the site of application of the light becomes blue in every rabbit (Fig. 3).

Three rabbits are given pentobarbital in a quantity sufficient to produce complete anesthesia. The blood pressure is recorded from the carotid. It is 80, 80, and 85 in each of the three animals. Infrared is applied to the abdomen. Trypan blue is injected intravenously. This dye localizes around the periphery of the area of skin treated and in the underlying portion of the intestines. The normal blood pressure in a second group of three rabbits is determined before the infrared irradiation is applied. Local anesthesia is used (1.0 c.c. of 1 per cent novocaine) for the operative procedure necessary to put the cannula into the carotid. The pressure in these three rabbits is as follows: 90, 90, and 95. Trypan blue localizes in the skin and in the intestines of each of these rabbits. These two groups of rabbits are controls for the above experiment and show that this dye will localize in the skin provided the pressure has not reached the abnormally low level of 50 mm. Hg.

The results of this experiment show that trypan blue may fail to localize and to concentrate about areas of injury in the skin of rabbits intoxicated with ethyl alcohol; however, this dye always localizes and concentrates in the intestines of each of these intoxicated animals.

DISCUSSION

The results of this experiment confirm the observations of Pickrell,[1] and Cressman and Rigdon[2] that alcohol intoxication may completely inhibit the development of an inflammatory reaction in the skin of the rabbit. The data suggest that this phenomenon does not result directly from the effects of alcohol upon either the leucocytes or the local blood vessels. The circulation in the skin apparently is markedly reduced in intoxicated rabbits, and as a consequence of this trypan blue and polymorphonuclear fail to localize in the skin of these animals. The fall in blood pressure, the rapid rate of the respirations, the cold, clammy skin all indicate that a diminution has occurred in the blood flow through the peripheral vessels.

The circulation in the intestinal tract during intoxication is not affected similarly to that of the skin. Capillary permeability here is increased, and leucocytes localize about foci of bacteria the same as they do in a normal rabbit. Edmunds and Gunn[6] state that large quantities of alcohol lower the blood pressure by weakening the vasoconstrictor centers and the heart muscle. In a series of experiments it has been shown that a leucocytic reaction fails to occur in the skin of rabbits with a low blood pressure.[3-5] The results of the present experiment are consistent with these observations upon capillary permeability and the development of local inflammatory reactions.

SUMMARY

Polymorphonuclear leucocytes fail to localize and to concentrate about staphylococci in the skin of rabbits intoxicated with ethyl alcohol when the blood pressure is abnormally low.

Leucocytes concentrate about staphylococci injected into the colon of the same intoxicated animal. This variation in the reaction in the skin and the colon may result from the lowered pressure produced by alcoholic intoxication. When the pressure is lowered, the skin becomes cold and clammy, suggesting that the peripheral circulation is markedly decreased or completely inhibited. The circulation in the intestines apparently remains intact.

Capillary permeability as shown by the localization of trypan blue following an intravenous injection is absent in areas of injured skin in rabbits intoxicated with ethyl alcohol. It is present, however, in the injured areas of intestines of the same intoxicated rabbit.

REFERENCES

1. Pickrell, K. L.: The Effect of Alcoholic Intoxication and Ether Anesthesia on Resistance to Pneumococcal Infection, Bull. Johns Hopkins Hosp. 63: 238, 1938.
2. Cressman, Ralph D., and Rigdon, R. H.: Capillary Permeability and Inflammation in Narcotized Rabbits, Arch. Surg. 39: 586, 1939.
3. Rigdon, R. H., Miles, Robert M., and Bland, Richard P.: The Effect of Low Blood Pressure Upon Capillary Permeability and Inflammation in the Skin of the Rabbit. Surgery 12: 302, 1942.
4. Wilson, Harwell, and Rigdon, R. H.: Experimental Studies on Capillary Permeability and Inflammation in the Skin of Rabbits Following the Sectioning of the Spinal Cord, Arch. Surg. 45: 416, 1942.
5. Rigdon, R. H.: Capillary Permeability and Inflammation in Rabbits With Staphylococcic Septicemia, Arch. Surg. 44: 129, 1942.
6. Edmunds, C. W., and Gunn, J. A.: Cushing's Pharmacology and Therapeutics, ed. 9, Philadelphia, 1928, Lea & Febiger, page 191.

ALCOHOLIC CIRRHOSIS OF THE LIVER*

A Clinical and Pathologic Study of 356 Fatal Cases Selected From 12,267 Necropsies

J. D. Kirshbaum, M.D.,† and Norman Shure, M.D.
Chicago, Ill.

SINCE 1826 when Laënnec adequately described cirrhosis of the liver, it has been observed and recognized, without much difficulty, both clinically and pathologically. However, the observation of increased connective tissue stroma in livers of varying weights, sizes, shapes, colors, and histologic architectures has provoked a certain amount of confusion in the nomenclature. As a result, in common usage today are such terms as atrophic cirrhosis, hypertrophic cirrhosis, multilobular cirrhosis, portal cirrhosis, biliary cirrhosis, Laënnec's cirrhosis, Hanot's cirrhosis, hobnail liver, alcoholic cirrhosis, gin drinker's liver, toxic cirrhosis, Wilson's disease, Banti's disease as well as many others.

Broadly, cirrhosis of the liver is merely a condition in which the connective tissue stroma is increased. Any stimulus to fibroblastic proliferation may eventually produce a well-marked cirrhosis. Mallory[1] lists four conditions under which the fibroblasts of the liver stroma proliferate:

1. To replace any which have been destroyed as a result of abscess, infarcts, syphilis, colon bacillus infection, etc.
2. Following mechanical injury (stretching), as in chronic passive congestion and bile stasis.
3. To organize fibrin, as for thrombi in sinusoids.
4. To form stroma for islands of regenerated liver cells and for tumor nodules.

It is apparent that the number of agents which may initiate the ultimate picture of cirrhosis is very large and for that reason the question "Is cirrhosis of the liver a disease entity?" is often raised.

The great majority of cirrhotic livers fall into four major groups and can be classified as follows:

1. Toxic cirrhosis
2. Biliary cirrhosis
   - a. Obstructive
   - b. Infectious
3. Pigment cirrhosis
4. Alcoholic cirrhosis
   - a. Periportal cirrhosis
     - I. Atrophic form (small liver)
     - II. Hypertrophic form (large liver)

*From the Department of Pathology, Woodlawn Hospital, and Cook County Hospital, and the Department of Medicine, University of Illinois.

Received for publication, August 13, 1942.

†Formerly of the Department of Pathology of the Cook County Hospital.

b. Fatty cirrhosis (hypertrophic form)
(Large amount of fat and little or no fibrosis)

Though this classification does not include every type of sclerotic liver known, many of which are exceedingly rare, it does encompass the commonly known types and does, more or less, conform to the nomenclatures and classifications of other investigators.[3-5]

Toxic Cirrhosis.—This occurs after necrotizing lesions of the liver parenchyma and may follow acute infections, such as acute yellow atrophy, repeated attacks of catarrhal jaundice, Weil's disease; or it may be the result of the ingestion of such drugs as cinchophen or arsenic.

Biliary Cirrhosis.—The obstructive type is associated with obstruction of the biliary passages by stone or tumor; the infectious type begins as an ascending cholangitis. These two types frequently co-exist.

Pigment Cirrhosis.—In hemachromatosis, often called "bronzed diabetes" because of the association of the typical coloring of the skin with glycosuria, the cirrhosis of the liver is merely one of the manifestations of a generalized condition. Either as a result of the liver-cell necrosis or as a result of the irritation of the iron pigment collected around the periphery of the lobule, there is an active macrophage and fibroblastic proliferation from the periportal connective tissue.

Alcoholic Cirrhosis.—The term "alcoholic cirrhosis" is an arbitrary one and, perhaps, not a very good one. To limit the diagnosis of alcoholic cirrhosis to those cases in which a definite history of chronic alcoholism is given, as Boles and Clark[6] do in their report of autopsy material from the Philadelphia General Hospital, we feel is too strict. The fatty cirrhosis is conceded by most investigators to be of alcoholic origin. The Laënnec cirrhosis is the type that has been called "alcoholic" for over a hundred years. There is a third type of alcoholic liver, the so-called "hypertrophic fatty form." It is large, tense, and loaded with fat. The liver is swollen and greasy; it may appear granular due to the subsurface lobules being distended with fat (see Fig. 1). This type of liver usually shows little or no sign of fibrosis and is observed in young people between 30 and 40 years of age. This form is usually larger than normal, smooth in contour, and weighs about 3,000 Gm. It may, however, be lobulated and nodular, depending upon the amount of connective tissue present. On cutting it is found to be greasy yet resistant.

Periportal cirrhosis is the typical portal cirrhosis which was described by Laënnec. The liver may be large or small, smooth or granular. The thought that it is larger in its early stage and becomes atrophic has been discarded. The noxious agent which initiates the cirrhosis is thought to enter the liver by way of the portal blood stream. The adjacent liver cells are damaged and connective tissue proliferation results. The newly proliferated connective tissue may become condensed and sclerotic, producing the small, hard, hobnail liver. Because of obstruction to the portal circulation, ascites develops, the spleen becomes engorged, the mucosa of the gastrointestinal tract becomes congested, and attempts at the establishment of a collateral circulation to compensate for the decreased venous bed are apparent. The presence of a caput medusae on the anterior abdominal wall and the frequent hemorrhages resulting from rupture of dilated

and varicose veins in the lower esophagus are typically products of this common type of cirrhosis.

The association of cirrhosis of the liver with alcohol is still a debatable point. Attempts at the production of cirrhosis experimentally with alcohol have not been successful, though it is believed that alcohol may be an accessory factor in the production of cirrhosis due to bacterial infection. In attempting to produce experimental cirrhosis by the use of agents which might possibly exist as

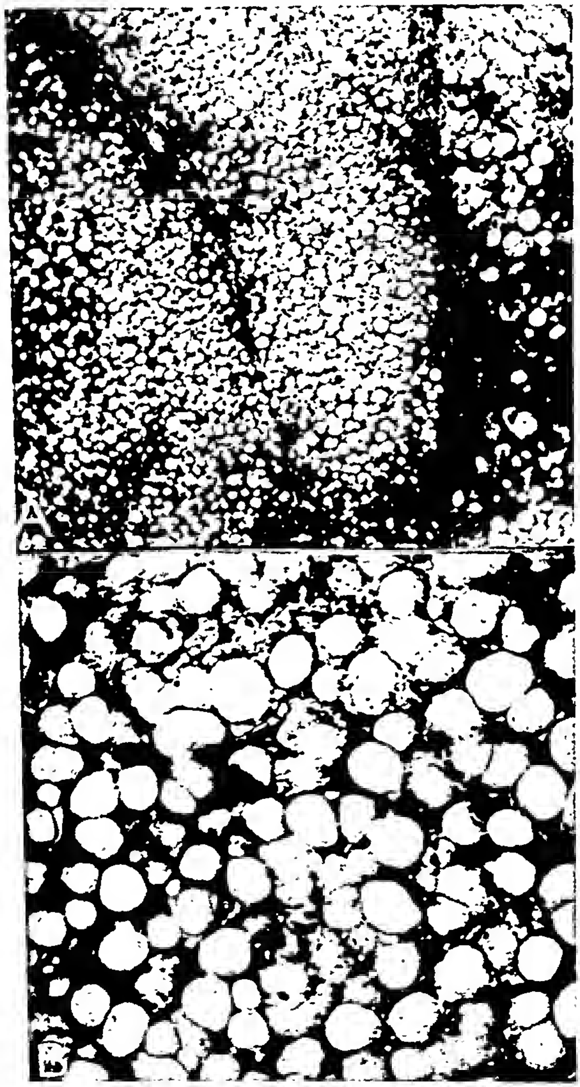


Fig. 1.—Photomicrograph shows (*A*) low-power field from a hypertrophic fatty liver. Note abundance of fat and very narrow periportal septa. *B*, High-power magnification. The liver cells contain large fat droplets and the nuclei are displaced to the periphery.

impurities in alcoholic beverages, Mallory was successful only with the use of copper. The recent work of Connor[7, 8] on the development of fatty livers in chronic alcoholism and the development of cirrhosis in fatty livers is interesting. Following the work of Himwick and Fazekas on liver metabolism in diabetes, Connor is of the opinion that in alcoholics there is a similar deficient carbohydrate

utilization. Because of the action of alcohol as a cell and tissue toxin there is a definite deficiency in carbohydrate metabolism and fat oxidation. In starvation or a carbohydrate-poor diet, such as is common in many alcoholics, sugar is withheld from the metabolic cycle. It has been shown that in experimental diabetes, in starvation, and in alcoholic intoxication the respiratory quotient of the liver is lower than that of the body as a whole or when fat alone is being consumed. In alcoholics it is probable that a condition similar to that of diabetes is produced and because of insufficiently utilized carbohydrates there is a piling up of unoxidizable fat within the liver cell.

The development of cirrhosis in a fatty liver, Connor states, is the result of pressure of the liver cells distended with fat upon the sinusoids. The sinusoids become collapsed and a thickening of the wall of the sinusoids is an early feature. The thickening of the walls with the formation of collagenous material promotes a reticulum production about the liver cells which have degenerated as a result of anoxemia. Fibroblastic proliferation follows. The interlobular stroma likewise is often stimulated to proliferate by the mechanical pressure of the distended liver lobules. Connor believes that the transition from a fatty liver to one of cirrhosis with very little or no fat is a result of the decreased carbohydrate intake an alcoholic assumes when his liver begins to provoke gastrointestinal symptoms.

Our material consists of the necropsy records of 356 cases of fatal alcoholic cirrhosis occurring in 12,267 consecutive autopsies performed at the Cook County Hospital from January 1, 1929, to July 1, 1939, inclusive.

Incidence.—There were 356 cases of cirrhosis, constituting 2.83 per cent of all our cases. This corresponds to Mallory's report of 2.2 per cent, but is higher than the incidence reported by Menne and Johnson,[9] or Evans and Gray.[10] It is somewhat lower than the incidence in Japan reported by Suzuki.[11] The yearly variation of cirrhosis of the liver, we believe, is significant (see Table I). Starting from a high level for 1929, there is a steady drop, reaching a low point in 1932. From 1933 there is a steady staggered rise. Though we are very reluctant to conclude from post-mortem records that a disease which may last ten or twenty years before resulting in death was less prevalent during a seven-year period, we cannot help noticing the regularity of the curve of yearly incidence. It can hardly be a coincidence that the curve is downhill until 1932 and has

TABLE I

INCIDENCE OF CIRRHOSIS

YEAR	AUTOPSIES	CIRRHOSIS	PER CENT
1929	955	28	2.93
1930	986	21	2.13
1931	1,114	25	2.24
1932	1,207	21	1.74
1933	1,192	31	2.60
1934	1,300	35	2.69
1935	1,176	40	3.40
1936	1,242	38	3.06
1937	1,167	52	4.45
1938	1,327	45	3.39
1939 (6 months)	601	20	3.35
Total	12,267	356	Avg. 2.83

been rising since. This peculiar level of incidence of a decrease until the year of the repeal of the prohibition amendment, followed by a steady rise, corresponds to the results of Evans and Gray[10] in their report of 17,874 autopsies at the Los Angeles General Hospital, and those of Boles and Clark[5] in their report of 4,000 autopsies at the Philadelphia General Hospital as well as the opinion of Rowntree,[12] who collected statistics from several institutions and public health agencies.

Sex.—Of our cases 244, or 68.5 per cent, were males, and 112, or 31.5 per cent, were females. Compared to our normal ratio of male to female autopsy material, which is two to one, we can conclude that cirrhosis occurs about twice as frequently in men as in women. These findings correspond with those of American observers, but are at odds with those of Japan and France. Suzuki,[11] in a report of 21 cases of cirrhosis out of 596 autopsies performed at the Kumamoto Medical College, found 19 males and 2 females. Paraf and Klotz[13] in a two-year period found that out of 1,030 females hospitalized at the Hôpital de Paris there were 23 cases of cirrhosis, or 2.2 per cent. In the same period, of 1,311 male admissions only 6, or 0.53 per cent, showed cirrhosis of the liver. The comparison, more than four times as frequent in females, is interesting and may open the way for a discussion on alcoholism in the French woman. The relation of sex to the occurrence of jaundice and ascites is noteworthy. The males in our group, though constituting 68.5 per cent of all our cases, comprised 76.4 per cent of all patients who had ascites alone. Conversely, the females comprised 39.5 per cent of all the cases having jaundice alone, somewhat above the figure for percentage of women having cirrhosis (see Table III).

Race.—There were 308, or 85.9 per cent, white and 50, or 41.1 per cent, colored persons in our series. Sex appeared to have no relation to race in our series, the male to female proportions being the same in both races. In our institutions where 35.8 per cent of our autopsy material consists of colored persons, the marked discrepancy of incidence is noteworthy.

TABLE II

DISTRIBUTION OF SEX AND RACE IN VARIOUS AGE GROUPS

	UNDER 30	31-40	41-50	51-60	61-	TOTAL
Male	1	22	68	89	64	244
Female	12	28	36	22	14	112
Colored	5	11	14	13	7	50
White	8	39	90	98	71	306
Total	13	50	104	111	78	356
Per cent	3.6	14.1	29.2	31.2	21.9	

Age.—Our youngest patient was 16 years; our oldest, 77 years. Thirteen, or 3.6 per cent, of our cases were under 30 years; 60, or 14.1 per cent, were 31 to 40; 104, or 29.2 per cent, were 41 to 50; 111, or 31.2 per cent, were 51 to 60; and 78, or 21.9 per cent, were over 60. The distribution of the various age groups in relation to sex and race can be seen in Table II. It appears that though 76, or 67 per cent, of the females were under 50, only 91, or 37 per cent, of the males were in the corresponding age group. The majority (64 per cent) of the males fall into the 41 to 60 age group while 57 per cent of the women are in the 31

to 50 age group. We believe that the increased incidence of cirrhosis in younger women in our series indicates mortality rather than pure incidence. Women with cirrhosis die at a younger age than do men. This is bolstered by the face that out of 13 cases in our youngest age group (under 30) 12 were women.

History of Alcoholism.—In 12 of our cases there was no clinical history available. Of the remaining 344 a definite admittance of chronic alcoholism was obtained in 144, or 41.9 per cent. We consider this to be far below the true incidence of indulgence in alcohol of the patients constituting our material. In obtaining the history of illness from the patient, the question of alcoholism is merely a part of the general survey of his habits. Unless a condition similar to cirrhosis or neuritis is suspected, the intern taking the history paid no more attention to the patient's temperance habits than he did to the amount of coffee or tea he drank or to the number of cigarettes he smoked. When a patient enters with obvious signs of intestinal obstruction, in diabetic coma, or with a perforated duodenal ulcer, as a great deal of our material does, the intern, with some justification, is not concerned with the patient's social habits and certainly does not anticipate the statistical survey which may result from his history. However, with the use of mimeographed forms for detailing a patient's history, our material is becoming more accurate and the only error remaining in a history of alcoholism will be that of the patient's reluctance in admitting it.

TABLE III

DISTRIBUTION OF JAUNDICE AND ASCITES

	JAUNDICE	ASCITES	JAUNDICE AND ASCITES	TOTAL JAUNDICE	NEITHER JAUNDICE NOR ASCITES
Male	26	65	87	113	66
Female	17	20	39	56	36
White	37	72	114	151	83
Colored	6	13	12	18	19
Under 30	4	1	3	7	5
31-40	8	7	21	29	14
41-50	13	21	46	59	24
51-60	16	31	35	51	29
Over 60	2	25	21	23	30
Total	43	85	126		102
Per cent	12.1	23.9	35.4		28.6

Jaundice and Ascites.—Jaundice occurred in 169, or 47.7 per cent, of our cases, and ascites in 211, or 59.2 per cent. The incidence of jaundice and ascites in relation to age, sex, and race is shown in Table III. It has already been pointed out that females are more likely to have jaundice without ascites and males are more likely to have ascites without jaundice. There are other significant facts apparent from Table III. Though the youngest age group constitute only 3.6 per cent of all the cases of cirrhosis, they comprise 9.3 per cent of all those having jaundice alone; the oldest age group, which make up 21.9 per cent of all our cases, constitute only 4.6 per cent of all cases of jaundice occurring alone. The incidence of jaundice alone shows a steady decrease as the age increases, while the occurrence of ascites alone increases with age. The combination forms (jaundice with ascites and neither jaundice nor ascites) seem to produce

opposing parabolic curves. Jaundice plus ascites is most prevalent in the middle age groups and is low in the extreme age groups: absence of both jaundice and ascites is lowest in the middle groups and highest in the extreme.

The ascites is produced by transudation of fluid from the mesenteric veins, a part of the portal system, as a result of obstruction and reduction in the size of the vascular bed. Our results of the occurrence of jaundice are at some variance with the common conception that jaundice does not occur frequently in this type of cirrhosis. Boyd says, in connection with the disturbed hepatic architecture seen, "Indeed it is to be wondered that jaundice is not a more marked feature in portal cirrhosis." Jaundice, with or without ascites, is most common in younger people. It occurred in 53.82 per cent of those under 30 years; 58 per cent of those 31 to 40; 56.7 per cent of those 41 to 50; 45.9 per cent of those 51 to 60; and 29.47 per cent of those over 60. We may interpret these results to indicate that jaundice is a late manifestation, late in the life of the disease rather than in the life of the patient. We may assume by the comparatively low incidence of jaundice in the older age group that these people survived because they did not develop jaundice, and that if they had developed jaundice at an early age they would have died at the earlier age to correspond with our increased incidence in the younger age groups.

Jaundice in cirrhosis is not due merely to compression of the bile ducts by connective tissue. It is probably due to the inability of the liver cells to excrete the bile pigment which is sent to the liver in normal amounts. The liver cells are abnormal, both early in the disease when the noxious agent which provokes connective tissue proliferation attacks them, and late in the disease when the increased connective tissue obstructs the sinusoids so as to produce venous stasis and subsequent anoxemia of the liver cells.

Esophageal Varices.—In our series there were 103 cases of esophageal varices constituting 28.6 per cent of the total (76 males; 27 females). Of these, in 33, or 32 per cent, of the number of cases with dilated and varicose esophageal veins, there were definite bleeding points noted. In many of these a massive hemorrhage was the immediate cause of death. This hardly corresponds with the report of McIndoe,[14] who notes a mortality due to hemorrhage of 50 per cent in 26 cases of advanced cirrhosis.

The incidence of esophageal varices in relation to age, as seen in Table IV, shows an increase with age until the 60-year-old group is reached, and is followed by a marked decrease. In examining the incidence of ruptured esophageal veins in the different sexes, we noted that though the females comprise 26 per cent of the total cases of varices and 20 per cent of those which have not ruptured, they constitute 39.3 per cent of the ruptured group. In addition, of the 27 women who had esophageal varices, 13, or 48.1 per cent, ruptured as compared to 20 of 76, or 26.3 per cent, of the men. This seems to indicate that though the presence of esophageal varices is somewhat less common in women than it is in men, when they do occur in women they are more likely to rupture.

Operations to produce more abundant anastomoses between the portal and systemic venous systems have been done repeatedly since 1889 when von der Meulen performed the first at the suggestion of Talma. After Morison performed the first successful one in 1895, varying reports of success have been

TABLE IV

ESOPHAGEAL VARICES IN RELATION TO SEX AND AGE

AGE	VARICES	MALE	FEMALE	TOTALS	
Under 30	Unruptured	0	1	1	3
	Ruptured	0	2	2	
31-40	Unruptured	6	1	7	13
	Ruptured	1	5	6	
41-50	Unruptured	15	7	22	34
	Ruptured	7	5	12	
51-60	Unruptured	20	4	24	34
	Ruptured	9	1	10	
Over 60	Unruptured	15	1	16	19
	Ruptured	3	0	3	

reported by Monprofit, Bunge, White, Mayo, and Tempsky. In a recent paper Grinnell[15] lists 23 cases in which 22 were followed up after the performance of surgery. He reports 9 per cent symptom free after a period of years; 32 per cent improved and 59 per cent unimproved. With an operative mortality of 27 per cent these figures are not encouraging.

TABLE V

BLOOD PRESSURE AND WEIGHT OF HEART

AGE	SYSTOLIC BLOOD PRESSURE					WEIGHT OF HEART		
	UNDER 120	120-140	140-160	160-180	OVER 180	UNDER 325 GM.	325-400 GM.	OVER 400 GM.
Under 30	4	4				13	0	0
31-40	12	11	7	2	4	23	10	11
41-50	28	23	20	4	4	48	35	21
51-60	29	16	25	15	7	37	29	31
Over 60	13	18	11	9	7	29	17	25
Total	86	72	63	30	22	170	91	88

Blood Pressure.—It is often stated that a persistently low arterial blood pressure is maintained in cirrhosis of the liver. Our material included cases of hypertension as well as those of hypotension. Of 273 cases in which a record of the blood pressure was available there were 158 in which the systolic arterial pressure was under 140 and 115 in which it was above 140. Table V indicates the distribution of the arterial tension in relation to the age of the individual; the weight of the hearts, an increase which often corroborates the clinical finding of hypertension, is also shown. In 179 cases the heart showed anatomical evidences of hypertrophy. There seems to be no indication that cirrhosis of the liver per se has any marked effect on the cardiovascular system.

Weight of the Liver.—In the 343 cases we recorded, 214, or 62.4 per cent, might be termed "hypertrophic"; 129, or 37.6 per cent, were "atrophic," and 66, or 19.2 per cent, were of the hypertrophic fatty form. The comparison of the incidence of the various liver weights in each age group is interesting (see Table VI).

TABLE VI

WEIGHT OF THE LIVER

AGE	UNDER 1,000 GM.	1,000-1,500 GM.	1,500-3,000 GM.	OVER 3,000 GM.	TOTAL
Under 30	1	2	7	3	13
31-40	4	11	28	7	50
41-50	8	28	54	13	99
51-60	5	36	55	10	106
Over 60	13	25	34	3	75
Total	31	98	178	36	343

Weight of the Spleen.—In one of our series a splenectomy had been done long before admission to the hospital; in four others the weight of the spleen was not recorded. Of the recorded weights the smallest was 50 Gm. and the largest was 2,045 Gm. Except for 12 the spleens were unanimously enlarged. Table VII shows the weights of the spleen in relation to sex and race. There is no marked difference in the sexes. In the races, however, there is a great variation. In 12 of 50 colored persons (24 per cent) the spleen weighed less than 100 Gm. as compared to 4 per cent in white patients. Sixty-two per cent of the colored persons had spleens weighing less than 200 Gm., while in 52.6 per cent of the whites it weighed more than 300 Gm. It is well known that the normal weight of the spleen in colored persons is less than in white, the discrepancy, however, in our results can hardly be explained on that basis. The abnormal constitution of the red blood cell in the Negro race is shown in its peculiar tendency toward sickling. We can only infer, at this point, that the marked difference in the weights of the spleens in our colored patients as compared to that in the white is merely another manifestation of the constitutional difference in the hematopoietic tissues of the Negro race.

TABLE VII

WEIGHT OF THE SPLEEN

WEIGHT	UNDER 100	100-200	200-300	300-400	400-600	OVER 600	TOTAL
Colored	12	19	7	4	8	0	50
White	12	65	66	62	68	28	301
Male	12	56	53	47	53	20	241
Female	12	28	20	19	23	8	110
Total	24	84	73	66	76	28	351

Pancreas.—There was no marked variation in the weights of the pancreas except in isolated cases, one weighing as much as 230 Gm., another as little as 30 Gm. In 129, or 36.2 per cent, of our cases, a well-marked fibrosis was noted.

There were five cases in which calculi were found in the pancreatic duct. This pancreolithiasis may be considered secondary to the cirrhotic condition of the pancreas. Cirrhosis was noted in 50 per cent of all cases of pancreolithiasis encountered in our necroptic material.

Carcinoma of the Liver.—There were 11 cases, or 3.09 per cent, of primary hepatocellular carcinoma of the liver as an incidental finding in our material. In the same period, however, there were 30 cases of primary malignant hepatoma; 11, or 37 per cent, had an associated cirrhosis. Both types of carcinoma, the massive discrete form and the multicentric form, were encountered. The in-

cidence of primary carcinoma in cirrhosis (3.09 per cent) and that of cirrhosis in primary carcinoma (37 per cent), we feel, corroborates the well-established conception that cirrhosis is the primary condition and that the tumor probably arises from the hyperplastic nodules. Worthy of note at this point is the incidence of primary carcinoma of the liver. Smith[16] in 1933, using part of our autopsy material, found 25 cases in 4,044 autopsies, an incidence of 0.56 per cent. We have collected from our material 30 cases in 12,267 autopsies, an incidence of 0.24 per cent.

Pectoral Alopecia.—Among numerous signs and symptoms connected with cirrhosis of the liver, two findings have received little attention: gynecomastia[17] and loss of axillary hair.[18] At the Cook County Hospital during the past ten years absence of hair on the chest in cases of cirrhosis has been emphasized. There has never been any formal effort made to corroborate this finding.

Of the 244 males in this series the presence or absence of hair on the chest was mentioned in necropsy protocols in 89. Seventy-six of these had no hair and 13 had sufficient hair to prompt such descriptions as "moderate amount of hair," "there is a coarse hairy growth," and "abundant hair on the chest." The interpretation of these statistics is variable. If we assume that the prosector, when doing the autopsy, mentioned the hair on the chest only when he happened to think of it, the incidence of pectoral alopecia is 76 out of 89 cases, or 85.2 per cent. If, on the other hand, the prosector mentioned the hair only when it was absent the incidence is 76 out of 244 cases, or 31 per cent. If the latter is true, it is difficult to explain the 13 cases in which the presence of hair was definitely noted.

SUMMARY

Three hundred and fifty-six fatal cases of cirrhosis of the liver were selected from 12,267 necropsies for study. In 184 cases cirrhosis was the primary anatomic cause of death, while in 172 cases the cirrhosis was an incidental finding. The cases were classified into two main groups: (1) periportal cirrhosis (atrophic or hypertrophic forms), and (2) fatty cirrhosis (hypertrophic form with a large amount of fat and little or no fibrosis). A total of 62.4 per cent of the livers were enlarged and 37.6 per cent were atrophic. Jaundice and hepatorenal decompensation were a frequent finding in the second group of cases (hypertrophic fatty form).

There were 68.5 per cent males and 31.5 per cent females. As to race, 85.9 per cent were white and 14.1 per cent were colored. Jaundice occurred in 169 cases, or 47.7 per cent, and ascites was present in 211 cases, or 59.2 per cent.

As to age, 104, or 29.2 per cent, occurred between 41 and 50, and 111, or 31.2 per cent, between 51 and 60 years. Hemorrhage from esophageal varices was noted in 33, or 9.2 per cent of the cases.

Hypertension was noted clinically in 32 per cent of the cases. However, a higher incidence existed (50.3 per cent) anatomically, considering hypertrophy of the heart as an index of a pre-existent essential hypertension.

Splenomegaly was a constant finding.

The pancreas was fibrosed in 36.2 per cent of the cases and in 5 cases concretions in the pancreatic ducts were found. Cirrhosis was noted in 50 per cent of all cases of pancreolithiasis.

Pectoral alopecia was observed at necropsy in 37 per cent of the males, a finding often of clinical diagnostic aid.

Hepatocellular carcinoma was noted as an incidental finding in 3.09 per cent of the cases, while 37 per cent of the cases of carcinoma of the liver showed cirrhosis.

REFERENCES

1. Mallory, F. B.: Cirrhosis of the Liver, New England J. Med. 206: 1231, 1932.
2. Hart, J. F., and Lisa, J. R.: Histogenesis of Laënnec's Cirrhosis, New York State J. Med. 37: 1619, 1937.
3. Albot, G.: Sur la classification anatomo-clinique des hépatites et des cirrhoses, Ann. d'anat. path. 13: 906, 1936.
4. Mallory, F. B.: Bull. Johns Hopkins Hosp. 12: 69, 1911.
5. Boyd, William: Text Book of Pathology, Philadelphia, 1938, Lea & Febiger.
6. Boles, R. S., and Clark, J. H.: Role of Alcohol in Cirrhosis of the Liver, J. A. M. A. 107: 1200, 1936.
7. Connor, L. C.: Etiology and Pathogenesis of Alcoholic Cirrhosis of the Liver, J. A. M. A. 112: 387, 1937.
8. Connor, L. C.: Fatty Infiltration and Development of Cirrhosis in Diabetes and Chronic Alcoholism, Am. J. Path. 14: 347, 1938.
9. Menne, F. R., and Johnson, T. W.: Cirrhosis of the Liver: Its Character and Incidence in 6,500 Autopsies, Northwest Med. 32: 129, 1933.
10. Evans, N., and Gray, P. A.: Laënnec Cirrhosis, J. A. M. A. 110: 1159, 1938.
11. Suzuki, K.: Contributions to the Pathology of Liver Cirrhosis, Especially the Statistical Pathological Report About the Dissected Cases in the Pathological Institute of the Kumamoto Medical College, Jap. J. M. Sc. Tr., V, Path. 2: 1, 1934.
12. Rowntree, L. G.: Discussion of Paper by Boles and Clark, J. A. M. A. 107: 1204, 1936.
13. Paraf, J., and Klotz, H. P.: Frequence actualle plus grande des manifestations alcoaliques chez la femme que chez l'homme, Bull. et mém. soc. méd. d. hôp. de Paris 49: 1373, 1933.
14. McIndoe, A. H.: Delayed Hemorrhage Following Traumatic Rupture of Spleen, Proc. Staff Meet., Mayo Clin. 4: 122, 1929.
15. Grinnell, R. S.: Omentopexy in Portal Cirrhosis of the Liver, With Ascites, Ann. Surg. 101: 891, 1935.
16. Smith, K. J.: Primary Carcinoma of the Liver, J. Lab. & Clin. Med. 18: 915, 1933.
17. Capriglione, L., Berardinelli, W., and da Costa Cruz, F.: Cirrhose Hepatique et Gynecomastie, Presse méd. 42: 1419, 1934.
18. Jacob, A.: Loss of Axillary Hair in Course of Cirrhosis, Presse méd. 42: 2076, 1934.

CLINICAL CHEMISTRY

SEX HORMONE ASSAYS IN THE MENOPAUSE THEIR CLINICAL SIGNIFICANCE*

B. A. Watson, M.D., N. Yolton, and L. Rahls
Battle Creek, Mich.

THE purpose of this communication is to report the results and possible clinical significance of estrogenic and 17-ketosteroid urinary assays of 28 women suffering from the "menopausal syndrome."

SUBJECTS

All patients presented some well-known signs and symptoms of the menopause. For purposes of study they were classified as: 1. Those patients that had either natural or artificial menopause. 2. Those with or without one of the most subjective of all menopause symptoms—"hot flashes."

PROCEDURE

At least two, and in some instances four, complete twenty-four-hour urine specimens were obtained on consecutive or alternate days until consistent daily estrogenic and androgenic values were obtained.

The urines were hydrolyzed and extracted by the Hershberg and Wolfe[1] method and were fractionated by the method of Gallagher and others.[2] Specimens were assayed for estrogenic values by injection in divided doses of an aqueous suspension into 15 ovariectomized mice. Values were reported in terms of gammas of estrone based on a standard dosage-response curve. 17-Ketosteroid assays were done by the Friedgood-Whidden[3] modification of the Zimmerman method, using a Cenco spectrophotelometer and reported as milligrams of androsterone.

Table I

	No. cases	Avg. age (years)	Mean estimate*	Range estimate	Mean androsterone†	Range androsterone
Natural menopause	16	51	6.8	1.1-22.9	9.59	3.7-20.8
Artificial menopause	12	46.8	6.75	2.9-15.0	8.75	4.4-14.25
Menopause with flashes	13	48.5	5.61	1.1-15.0	8.75	4.4-14.25
Menopause without flashes	15	49.6	7.68	1.5-22.9	9.58	3.7-20.8

*Gammas estrone.
†Milligrams androsterone.

DISCUSSION

Table I shows that there is a relationship between the mean values of the female and male sex hormone and symptoms; e.g., patients that did have "hot

*From the Endocrine Research Clinic of the Battle Creek Sanitarium.
Read by title before the Annual Meeting of the Association for the Study of Internal Secretions, May, 1941.
Received for publication, July 6, 1942.

flashes" had lower mean estrogenic values than those that did not exhibit this phenomena. This is not in accord with the observations of Fluhmann and Murphy,[4] who found there was no correlation between symptoms and the estrone content of the blood in the menopause patients.

Other interesting observations during this study were:

1. Some patients with severe menopausal symptoms were found to have female sex hormone values considerably above our normal values in the presence of a normal amount* of male sex hormone (Case 1).

2. Other patients had male sex hormones in the lower limits of normal and female sex hormones at the upper limits of normal.

3. Certain patients had male sex hormones at the upper limits of normal and female sex hormones at the lower limits of normal.

Our observations during this study: the fact that Salmon,[5] and Shorr et al.[6,7] have shown that testosterone propionate in large doses can relieve menopausal symptoms and that all clinicians have observed certain menopausal patients who will not respond to adequate estrogenic therapy, suggests a theory that possibly the menopausal syndrome may be due to:

A. A lack of female sex hormone (Case 2).

B. A lack of male sex hormone (Case 1).

C. Sex hormone imbalance within normal limits, e.g., (1) Low normal male, high normal female (Case 3); or (2) high normal male and low normal female sex hormone (Case 4).

Several other interesting cases which will be reported in detail later were observed. One woman, who five years previously had had a complete hysterectomy and ovariectomy by a competent surgeon, excreted estrone in amounts far above the upper limit of normal. Another patient who had normal amounts of estrone and androsterone was found to have a vaginal smear indicative of marked estrogenic deficiency. She was given adequate estrogenic therapy by her local physician until a normal vaginal smear was attained with no clinical improvement in symptoms.

TREATMENT

The results of the urinary assays were used empirically to guide hormonal treatment. Patients in group A were given female sex hormone. Patients in group B were given male sex hormone. Patients in group C_1 were given male sex hormone and those in group C_2 were given female sex hormone. Some patients with normal female and male sex hormones were given female sex hormone and served as controls (Case 5). Space does not permit publication of all case histories, but the following are examples of types of cases observed:

CASE 1.—Female, aged 40 years. Complaints: Marked nervousness, headache, excitability, fatigue, and loss of libido following an artificial menopause three years previously, with an average excretion of 22.9 gammas of estrone and 8.1 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Testosterone propionate was given in two courses of 10 mg. every four days for six weeks, with a rest period of two weeks in between courses.

---

*On a basis of as yet unpublished observations by Samuels, Yolton, and Winther at the University of Minnesota, arbitrary normal values for female sex hormones were assumed to be from 2.5 to 7.5 gammas of estrone and from 5 to 15 mg. of androsterone per twenty-four-hour specimen at the midmenstrual period. Thus, in those menopausal patients still menstruating specimens were collected at the middle of the cycle.

Results: Decided improvement in stability of equilibrium, loss of headache, definite improvement in fatigue, and a gain of 15 pounds in weight.

CASE 2.—Patient aged 51 years. Complaints: Hot flashes, sinking sensations, excess perspiration, feeling of intense heat, and irregular menses. Average excretion of 2.25 gammas of estrone and 7.59 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Progynon 10,000 units intramuscularly was given three times a week for two weeks, and two times a week for the next two weeks.

Results: Flashes practically disappeared as did most of the subjective symptoms.

CASE 3.—Patient aged 50 years. Complaints: Occasional hot flashes, marked periodic "migraine" headache, beginning shortly after cessation of menses at age 45. Stiffness of hands, back, and knees. Average excretion 6.6 gammas of estrone and 4.4 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Two courses of testosterone proprionate, 25 mg. every five days for six doses, with a two weeks' rest period between courses. After this therapy the patient has been able to maintain good health with 10 mg. every twelve days.

Results: Marked diminution in joint pain and almost complete relief from headaches.

CASE 4.—Patient aged 48 years. Complaints: Artificial menopause of two years' duration, moderate depression, loss of libido, hot flashes, fatigue, and emotional instability. Average excretion 4.35 gammas of estrone and 14.25 mg. of 17-ketosteroids.

Treatment: Ethinyl estradiol (Schering) in doses of 0.15 mg. a day.

Results: Improvement of emotional instability and depression, increased sense of well-being, and reduction of hot flashes from ten to one daily.

CASE 5.—Patient aged 45 years. Complaints: Hot flashes, excessive perspiration, increased nervousness, irritability, and fatigue following hysterectomy and partial ovariectomy three years previously. Average excretion 4.1 gammas of estrone and 5.35 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Patient insisted on hormonal therapy though there was no indication of a deficiency as judged by urinary assay.

Results: No improvement.

Case 1 demonstrates a patient with a comparatively low male, high female sex hormone ratio. *Case 2* is a patient with a deficiency of female sex hormone and adequate male sex hormone. *Case 3* shows a patient with adequate female sex hormone but a male sex hormone which is below normal. *Case 4* is a patient with a high male, low female sex hormone. *Case 5* is an example of one of our control patients with normal male and female sex hormones, adequately treated with female sex hormone with no results.

The results of treatment carried on during the seven months of this study, using urine assays as an empirical guide to therapy, have been gratifying in that treatment has been successful in most instances, and it is felt that the sex hormone assay has pointed the way to successful treatment of some patients and prevented the needless expense of therapy in others who need psychiatric rather than endocrine management.

SUMMARY

Routine determinations of the male and female sex hormones in the urines of menopausal patients may have clinical application in that (a) it may indicate whether male or female sex hormone therapy is needed; (b) aid in explaining a lack of response of certain menopausal patients to the usual estrogenic hormone therapy.

A disproportionate amount of either male or female sex hormones may in some patients cause certain menopausal symptoms which may be alleviated by proper endocrine therapy.

A correlation between the incidence of definite "hot flashes" and the estrogenic and androgenic content of the urine appears to exist.

Estrone has been found in the urines of patients who have had their ovaries and uterus removed.

Correlation of urinary sex hormone assays with clinical results in treatment of the menopause is indicated.

REFERENCES

1. Hershberg, E. B., and Wolfe, J. K.: Rapid Extraction for Urinary Steroids, J. Biol. Chem. 133: 667, 1940.
2. Gallagher, T. F., Peterson, D. H., Dorfman, R. I., Kenyon, A. T., and Koch, F. C.: Daily Urine Excretion of Estrogenic and Androgenic Substances by Normal Men and Women, J. Clin. Investigation 16: 695, 1937.
3. Friedgood, H. B., and Whidden, H. L.: Determination of Crystalline Androsterone With Evelyn Photoelectric Colorimeter, Endocrinology 25: 919, 1939.
4. Fluhmann, C. F., and Murphy, K. M.: Estrogenic and Gonadotropic Hormones in Blood of Climacteric Women and Castrates. Am. J. Obst. & Gynec. 38: 778, 1939.
5. Salmon, U. J.: Effect of Testosterone Proprionate Upon Gonadotropic Hormone Excretion and Vaginal Smears of Human Female Castrate, Proc. Soc. Exper. Biol. & Med. 37: 488, 1937.
6. Salmon, U. J.: Gonadotropic Effect of Androgens Upon Immature Rat Ovary, Proc. Soc. Exper. Biol. & Med. 38: 352, 1938.
7. Shorr, E., Papanicolaou, G. N., and Stimmel, B. F.: Neutralization of Ovarian Follicular Hormone in Women by Simultaneous Administration of Male Sex Hormone, Proc. Soc. Exper. Biol. & Med. 38: 759, 1938.

A NOTE ON THE BLOOD GUANIDINE LEVEL IN MIGRAINE SUBJECTS*

HAROLD D. PALMER, M.D., D. B. MC NAIR SCOTT, PH.D., AND
K. A. C. ELLIOTT, PH.D.
PHILADELPHIA, PA.

SINCE migraine headache is held by some authorities to be related to essential epilepsy, it was of interest to determine whether changes in blood guanidine occur in connection with migraine attacks, as Murray and Hoffmann[1] have found to be the case in connection with epileptic seizures. These latter reported that the concentration of guanidine-like substances in the blood of patients with essential epilepsy rose considerably during the aura preceding seizures, reached a high level during the convulsion, and returned to the basic level within an hour thereafter. It was also of interest to check by our technique the basal blood guanidine levels in epileptics and the levels in a group of patients of mixed pathology. A group of normals was tested as a control.

Table I shows the results obtained in this study. Differences between the averages for the groups tested were not significant. Migraine patients showed no

*From the Institute of the Pennsylvania Hospital, Philadelphia, and the Department of Psychiatry of the Woman's Medical College of Pennsylvania.
Received for publication, October 13, 1942.

TABLE I

BLOOD GUANIDINE-LIKE SUBSTANCES

Milligrams per 100 c.c.

NORMAL SUBJECTS	MIGRAINE PATIENTS*		EPILEPTICS BASAL	OTHER PATIENTS	
	BASAL	DURING ATTACK			
0.40	0.41	0.40	0.50	0.18	Depressed
0.47	0.45	0.54	0.43	0.44	Depressed
0.48	0.47	0.54	0.35	0.37	Depressed
0.50	0.51	0.40	0.41	0.40	Anxiety
0.40	0.40	0.51	0.40	0.53	Narcolepsy
0.48	0.30	0.60		0.33	Hypothyroid
0.48	0.61	0.49		0.40	Histamine headache
0.42	0.51	0.55			
Averages					
0.45	0.46	0.50	0.42	0.42	

*The figures under "basal" and "during attack" were obtained on different patients.

material change in blood guanidine in connection with attacks, and the basal levels in epileptics were normal, as were the levels in the group of mixed pathology.

By comparison of their figures for epileptics with those reported for normal subjects by Andes and Myers[2] (0.21-0.28 mg. per 100 c.c.), Murray and Hoffmann concluded that the basal levels in the epileptics studied by them were higher than normal. Our basal values for epileptics are approximately the same as Murray and Hoffmann's and do not differ materially from our figures for normals. They are higher than the normal level found by Andes and Myers. This discrepancy is possibly caused by the differences in technique used in measuring the color developed in the determination. The photoelectric method used by Murray and Hoffmann and ourselves may give higher values than the ocular method used by Andes and Myers.

The groups studied were composed of about equal numbers of men and women. The persons with migraine, all of whom suffered from typical hemicrania of long standing, were patients of the Migraine Clinic of the Institute of the Pennsylvania Hospital, or were persons connected in some capacity with the Institute. The epileptics were patients resident in the Pennsylvania Hospital, Department for Mental and Nervous Diseases, or in the Philadelphia General Hospital. The normal subjects were obtained from the medical, laboratory, and nursing staffs of the Institute. The group of mixed pathology were from the private practice of one of us.

Blood guanidine derivatives were determined in duplicate by the method of Andes and Myers, except that the color was finally developed in a total of 5 c.c. instead of 2.5 c.c. and was measured by the Klett-Summerson photoelectric colorimeter, using a No. 540 filter.

SUMMARY

1. No sign of abnormality or material change was found in the concentration of guanidine derivatives in the blood of migraine patients previous to or during attacks.

2. The basal level of guanidine-like substances in a number of epileptics was not higher than the level in normal subjects.

REFERENCES

1. Murray, M., and Hoffmann, C. R.: The Occurrence of Guanidine-Like Substances in the Blood in Essential Epilepsy, J. Lab. & Clin. Med. 25: 1072, 1940.
2. Andes, J. E., and Myers, V. C.: Guanidine-like Substances in the Blood. I. Colorimetric Estimations and Normal Values, J. Lab. & Clin. Med. 22: 1147, 1937.

THE APPARENT FRUCTOSE CONTENT OF HUMAN EXTRACELLULAR FLUID*

Roger S. Hubbard, Ph.D., and Emerson Holley, M.D.
Buffalo, N. Y.

IN EARLIER publications[1-3] the presence in human spinal fluid of fructose, or of a compound with properties closely resembling those of fructose, has been reported. This substance was found in much greater concentration in spinal fluid than in blood. It has seemed desirable to study human extracellular fluid obtained from sources other than the spinal canal to determine whether this rather unexpected finding is a general or a specific one. In the present note the results of a series of such studies are presented.

The material used in the present study was obtained from medical wards of the Buffalo General Hospital and the Edward J. Meyer Memorial Hospital. A few analyses were made upon specimens obtained at post-mortem examinations made by the department of pathology of the Buffalo General Hospital. Since edema fluid approximates cerebrospinal fluid in its chemical composition, several samples of this material were studied. These were obtained by inserting small metal tubes—the so-called "Southey tubes"—under the skin of patients having gross accumulations of fluid whenever it was possible to do so. It seemed desirable also to study fluids which were rich in protein. Specimens of this nature were obtained either at autopsy or by puncture and aspiration from various portions of the bodies of living patients. The patients for the most part were not fasting when the specimens were drawn. As far as could be determined all the specimens studied were sterile. Blood was taken at the same time as were the specimens of fluid.

The technical methods used were the same as those previously described.[2] Protein and probably other interfering compounds were removed with zinc sulfate and sodium hydroxide in the proportions recommended by Somogyi.[4] When the protein concentration was low, it was possible to prepare suitable filtrates with a minimum dilution of the initial material, but when blood was analyzed, or when the fluids studied were rich in protein, it was necessary to make dilutions which were frequently as great as 1 to 10. The concentrations of zinc and sodium hydroxide used were the same in all instances; only the relative volumes of the sample and of distilled water varied. Final analyses were made, as described in previous articles.[2] Reducing compounds were determined by the method of Folin and Wu,[5, 6] modified, when the concentrations were very

*From the University of Buffalo, Medical School, and the Buffalo General Hospital and Edward J. Meyer Memorial Hospital.

Received for publication, November 5, 1942.

low, according to the recommendations of Hubbard and Allison.[7] Fructose, and fructose-like compounds were determined by the resorcinol technique of Roe.[8] Glucose gives a color when treated with the reagents used by Roe, and it was, therefore, necessary to correct the values obtained for the error produced by the presence in all samples of relatively large amounts of this sugar. Two methods were used for this purpose during the study reported. In some instances the correction was based upon analyses of many solutions of pure glucose carried through Roe's technique. In other experiments an analysis was first made by the technique of Folin and Wu, and a solution was prepared which contained the same concentration of pure glucose as did the specimen of blood or fluid analyzed. This solution of glucose was carried through the procedure simultaneously with the analysis of the unknown solution, and read against a standard solution of pure fructose. By subtracting the value so obtained from the reading given by the sample, the corrected fructose content of the blood or fluid was determined. In a number of instances an attempt was made to determine the effect of a short period of fermentation upon the apparent sugar content of the fluids.[9-11] For this purpose suspensions of washed baker's yeast cells were added to the blood and fluid to give concentrations of 1 c.c. of cells in 5 c.c. of fluid. These stood half an hour to an hour at room temperature, and were then precipitated and analyzed as described above.

Table I contains the results found. Only the corrected values for fructose are given. Because the accuracy of the fructose determinations in fluid were much greater when the filtrate analyzed was only slightly diluted, the degree of dilution is given in each instance. The determinations of "fructose" content after fermentation are included, but the nonglucose reducing compounds, which appeared to add little to the value of the data, have been omitted. The average values of these substances were equivalent to 6 and 8 mg. of glucose per 100 c.c. of fluid and blood, respectively. In only one instance did a study appear unsatisfactory. Difficulty was experienced in removing protein from the abdominal fluid in Experiment 27, and it was necessary to reprecipitate with a second addition of zinc and sodium hydroxide in this instance. Whether this procedure affected the significance of the results could not be determined.

We believe that a number of interesting facts are shown by Table I. The average concentrations of glucose in the blood and fluid specimens were approximately the same—99.0 mg. per 100 c.c. in the former, and 103.7 mg. in the latter material. In a majority of the specimens, however, the concentration was higher in the extracellular fluid than in the blood. This difference was not due to the presence of nonfermentable reducing compounds in the specimens of fluid analyzed. It probably resulted from a number of causes. These include the relatively greater water content of the fluids, and the relatively low glycolysis which results from the low concentrations of cells which most of them contained. (Note that in the specimens of synovial fluid rich in cells the glucose content was unusually low.) It is possible also that an uneven distribution of sugar between the blood and the fluid, similar to that which has been reported in spinal fluid,[12] may have influenced the results, for most of the fluids were not taken in a postabsorptive state, and the findings may represent an equilibrium attained when the blood sugar concentration was high shortly after a meal.

TABLE I

NUMBER	TYPE OF FLUID	ANALYSES MADE BEFORE FERMENTATION					AFTER FERMENTATION		NOTES
		BLOOD		EXTRACELLULAR FLUIDS			BLOOD	FLUID	
		Glucose (mg./100 c.c.)	Fructose (mg./100 c.c.)	Dilution	Glucose (mg./100 c.c.)	Fructose (mg./100 c.c.)	"Fructose" (mg./100 c.c.)	"Fructose" (mg./100 c.c.)	
1	Edema	77	0.0	1:3.6	101	0.4	-	-	-
2	Edema	75	0.5	1:1.2	97	0.5	-	-	-
3	Ascitic	-	-	1:5	117	1.8	-	-	-
4	Pericardial	-	-	1:3	109	1.5	-	-	Obtained at post mortem
5	Pericardial	-	-	1:3	177	0.0	-	-	Obtained at post mortem
6	Pericardial	-	-	1:3	80	0.3	-	-	Obtained at post mortem
7	Ascitic	-	-	1:5	142	0.0	-	-	-
8	Ascitic	181	-	1:3	182	0.0	-	-	-
9	Edema	-	-	1:2	120	0.7	-	0.0	Same case as No. 10
10	Edema	-	-	1:5	93	0.5	-	-	Same case as No. 9
11	Pericardial	-	-	1:3	135	0.0	-	0.0	Obtained at post mortem
12	Pericardial	-	-	1:3	117	0.0	-	0.0	Obtained at post mortem
13	Edema	85	0.5	1:5	97	0.6	-	-	-
14	Pleural	87	0.8*	1:2	93	0.8*	0.0	0.0	-
15	Edema	99	2.8*	1:2	116	3.0*	1.5	1.8	-
16	Pleural	134	0.2*	1:2	160	0.2*	0.4	0.4	-
17	Edema	77	2.8*	1:2	98	2.7*	1.7	1.6	-
18	Pleural	112	0.9*	1:5	127	1.3*	-	1.5	-
19	Synovial	152	1.0*	1:10	67	0.3*	-	-	Rich in cells; same case as No. 23
20	Ascitic	85	1.2*	1:5	116	1.2*	-	-	-
21	Pericardial	108	0.2*	1:12	6	0.0*	-	-	Gross blood present
22	Edema	76	0.4*	1:2.4	92	0.2*	1.0	0.2	-
23	Synovial	95	1.5*	1:10	63	1.0*	0.2	0.8	Rich in cells; same case as No. 19
24	Ascitic	131	1.0*	1:10	131	1.0*	-	-	-
25	Ascitic	94	1.3*	1:10	115	0.0*	-	-	-
26	Pleural	117	3.0*	1:10	102	0.8*	-	-	-
27	Ascitic	108	0.9*	1:2.4	142	1.7*	0.6	0.5	Unsatisfactory filtrate (see text)
28	Pleural	108	1.0*	1:5	138	0.5*	0.0	0.6	Chyliform fluid
29	Edema	83	1.1*	1:10	63	0.0*	-	-	Fluid rich in protein
30	Edema	101	0.5*	1:2	110	0.2*	0.3	0.1	-
31	Edema	100	0.6*	1:2	110	0.4*	0.7	0.2	-
32	Edema	59	2.0*	1:2	85	1.3*	-	-	Same case as No. 33
33	Edema	53	0.3*	1:2	74	0.1*	-	-	Same case as No. 32

Under glucose are given the concentrations of total reducing compounds expressed as glucose.
Under fructose are given the concentrations of substances reacting with resorcinol expressed as fructose.
The filtrates prepared from blood represented a 1:10 dilution of the initial material.
Fructose values have been corrected for the reaction given by the glucose present. The correction used for the starred (*) values were based upon determinations run simultaneously with the analyses; for others a standard correction was determined in preliminary experiments.

Sugar may have been removed from the blood by active metabolic processes, and a complete re-establishment of conditions of equilibrium by diffusion not have been reached. It is interesting to note that in a series of analyses of blood and spinal fluid made by identical methods, glucose was regularly present in the spinal fluid in lower concentrations than in the blood.

The results of the fructose determinations were also interesting. The average value of the corrected "fructose" concentrations in all the 33 fluid specimens studied was 0.7 mg. per 100 c.c. This differs markedly from the results upon spinal fluid specimens previously reported, for the average found upon 32 specimens reported previously was 2.9 mg. per 100 c.c. The contrast between the individual specimens was as marked as was the difference between these averages. In only 8 fluid specimens was the concentration greater than 1 mg. per 100 c.c., while only four spinal fluid specimens contained concentrations which were less than this. Each of these four specimens was obtained from a patient with meningitis, and there seemed to be good reasons for associating the low values with the clinical condition of the patients.[3] In 26 of the 32 spinal fluid specimens the concentration of fructose was greater than 2 mg. per 100 c.c., while there were only two results in the present study which contained such amounts of fructose. It seems obvious, therefore, that there is a marked difference between the apparent fructose content of cerebrospinal fluid and other types of extracellular fluid.

The data were examined to determine whether there were any differences between values upon fluids obtained from different body cavities. As far as could be determined there were no such differences. The highest fructose concentration found was in a specimen of edema fluid, but another specimen apparently contained none of the sugar, and in several others the concentrations were of the order of 0.1 or 0.2 mg. per 100 c.c. As a comparison with peripheral edema fluid, which is almost regularly very low in protein, a number of specimens of pericardial fluid, in which the content is regularly high, were analyzed. The results of these analyses also covered a wide range, from 1.5 to 0.0 mg. per 100 c.c. The rather limited number of other types of fluid, chest fluid, abdominal fluid, and synovial fluid, all showed similar variations.

It seemed possible that the method of handling the specimens might have affected the results. As already stated, when the protein content was high, it was necessary to dilute the material in order to obtain a filtrate suitable for analysis; when the protein concentration was low, marked dilution was unnecessary. The average of the values obtained when specimens were diluted five or ten times was 0.65, and when diluted less than five times it was 0.76 mg. per 100 c.c. Such variations in technique, therefore, had no significant effect upon the results. It is certain, however, that the probable significance of any single figure obtained when the more concentrated filtrates were analyzed was greater than when dilution was necessary.

A comparison between the fructose content of blood and of the various fluids was made. In the 23 studies in which results of both determinations were available the average fructose content of blood was 1.1, and of the fluids, 0.8 mg. per 100 c.c. This relationship is quite different from that observed in the study of

spinal fluid, for in that investigation the average fluid content of fructose was approximately six times as great as was the concentration in blood. If the figures presented in Table I are examined in detail, it is evident that results upon individual subjects agree at least as closely together as do the average ones, for in only a third of the studies do the results differ by 0.5 mg. per 100 c.c. or more. In all but one of the analyses in which the differences were of this magnitude, both the blood and the fluid were diluted 1:10, and the errors of both determinations were great.

Because there was so little difference between the average blood and fluid values, it seemed worth while to calculate the correlation coefficient between the two sets of figures. This had been done in the study of spinal fluid, and no significant correlation was found. In this instance the coefficient was +0.64 ± 0.08. These figures make it seem probable that the fructose, or fructose-like compound, found in the extracellular fluids is derived from the blood, or at least is in equilibrium with some similar materials in the blood.

In the discussion of the results obtained in the previous study[2] it was pointed out that it was possible that no fructose is present in blood, because the accuracy of the determinations is so low. It seems to us that the correlation which appears to exist between the fructose in blood and extracellular fluid is evidence of the actual existence of some similar compound in both media.

An attempt was made to determine whether the material giving the color with resorcinol was actually fructose. In 14 experiments the fluid was inoculated with massive suspensions of yeast cells, allowed to incubate, and the apparent fructose content was determined. The results could not be considered satisfactory. In all but 4 of these specimens there was some apparent destruction of "fructose," and in some instances the destruction was marked. There was frequently noted, however, a measurable color when the incubated material reacted with the resorcinol reagents. The results with blood were essentially similar. There are at least three possible explanations for this finding. It is possible that at least two different substances in blood and extracellular fluid react with the resorcinol reagent, and that only one of these is readily destroyed by such suspensions of baker's yeast as were used in these experiments. The second possibility is that some fructose may have escaped destruction by the yeast suspensions used. It was found that some yeast suspensions do not ferment fructose readily, and it is possible that such small amounts of fructose as were apparently present in these solutions may have escaped destruction. The third possible explanation of the results is an extraction from yeast cells of some compound which will react with the resorcinol reagent. A suspension of yeast cells in distilled water has been incubated and analyzed as a part of each study presented in the table. These control suspensions regularly showed no color upon analysis. It was found, however, that certain alkaline solutions do extract from yeast cells measurable amounts of some compound which gives the resorcinol reaction, and it does not seem impossible that the blood and fluids may have had a similar action upon the yeast cells. It is, therefore, impossible to decide the exact nature of the compound which gave the resorcinol reaction in the unfermented blood and fluid specimens. It may all have been fructose; it may have

been some compound which resembles fructose, but which is slowly fermented by or adsorbed upon yeast cells: there may have been two compounds present which react with the resorcinol reagent, only one of which is acted upon by suspensions of washed cells of baker's yeast.

CONCLUSION

Results of the determination of the apparent fructose content of abnormal human extracellular fluid obtained from various body cavities have been carried out. Traces of some compound giving a reaction with the resorcinol reagent were present in two-thirds of the specimens. The amounts were much smaller than those previously found in cerebrospinal fluid. They appeared to be derived from, or to be in approximate equilibrium with, similar substances in blood. Fermentation with resting yeast cells was carried out in an attempt to identify the compound definitely as fructose, but the identification was not made with certainty.

REFERENCES

1. Hubbard, R. S., and Garbutt, H. R.: Resorcinol (Fructose) Reaction in Cerebrospinal Fluid, Proc. Soc. Exper. Biol. & Med. **32**: 986, 1935.
2. Hubbard, R. S., and Russell, N. M.: Fructose Content of Spinal Fluid, J. Biol. Chem. **119**: 647, 1937.
3. Hubbard, R. S., Russell, N. G., Jr., and Russell, N. M.: Sugars and Glycolytic Enzymes of Spinal Fluid in Epidemic Cerebrospinal Meningitis, J. Clin. Investigation **15**: 519, 1936.
4. Somogyi, M.: Method for Preparation of Blood Filtrate for Determination of Sugar, J. Biol. Chem. **88**: 655, 1930.
5. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. **38**: 81, 1919.
6. Folin, O., and Wu, H.: System of Blood Analysis; Simplified and Improved Method for the Determination of Sugar, J. Biol. Chem. **41**: 367, 1920.
7. Hubbard, R. S., and Allison, C. B.: Residual Reduction in Blood Filtrate After Treatment With Colon Bacillus, Proc. Soc. Exper. Biol. & Med. **25**: 408, 1928.
8. Roe, J. H.: Colorimetric Method for the Determination of Fructose in Blood and Urine, J. Biol. Chem. **107**: 15, 1934.
9. Hiller, A., Linder, G. C., and Van Slyke, D. D.: Reducing Substances of Blood, J. Biol. Chem. **64**: 625, 1925.
10. Somogyi, M.: Reducing Non-sugars and True Sugars in Human Blood, J. Biol. Chem. **75**: 33, 1927.
11. Benedict, S. R.: Determination of Blood Sugar, J. Biol. Chem. **76**: 457, 1928.
12. Fremont-Smith, F., and Darley, M. E.: Cerebrospinal Fluid Sugar, Arch. Neurol. & Psychiat. **14**: 390, 1925.

LABORATORY METHODS

GENERAL

A STERILITY TEST FOR SULFANILAMIDE POWDERS*

Maurice Landy, Ph.D., and Elizabeth J. Oswald, B.S.
Washington, D. C.

THE report of a death from tetanus traced to a contaminated sulfonamide powder[1] makes it evident that there is a present need for an adequate test to determine the sterility of these valuable chemotherapeutic agents. There would appear to be no question regarding the necessity of subjecting the powders to heat sterilization,[2, 3, 4] but there seems to be some controversy as to the amount of heat which can be applied without altering the drugs to such an extent as to render them useless and, in some cases, actually dangerous to administer.[5, 6] The margin of safety between the amount of heat necessary to kill any organisms present, and the amount which will yield a product unfit for use is comparatively small. It is therefore important to have available a test for the determination of the sterility of sulfonamide powders.

The procedure which has been used successfully in this laboratory is as follows: The contents of a shaker package (5 grams of sulfanilamide) are transferred aseptically to a flask containing 500 c.c. of sterile water. The flask is vigorously shaken for five minutes, allowed to stand for thirty minutes at room temperature, and shaken again for five minutes. The undissolved sulfanilamide is allowed to settle and 5 c.c. of the supernatant fluid are added to each of two tubes containing 20 c.c. of Brewer's medium.[7] The tubes are incubated at 37° C. for a period of seven days and examined for evidence of growth.

In the development of the above procedure it was necessary to select a medium capable of supporting both aerobic and anaerobic growth, and which would, in addition, nullify the bacteriostatic effect exerted by the sulfonamide under test.

Initial experiments demonstrated that Brewer's medium fulfilled these requirements. It contains a sufficient amount of sulfonamide-inhibitor to permit growth of three selected test organisms (*Staphylococcus aureus*–Pure Food and Drug strain, and Army Medical School strains of *Escherichia coli* and *Clostridium tetani*) in the presence of 50 mg. of sufanilamide. This amount of sulfanilamide is the maximum amount which could be present per tube of Brewer's medium in the execution of the aforegoing sterility test. Inasmuch as Brewer's medium, by assay,[8] contains 2 micrograms of p-aminobenzoic acid per 20 c.c., with the possible presence of additional sulfonamide-inhibitors, it

*From the Division of Bacteriology, Army Medical School.
Received for publication, December 30, 1942.

seems to be adequate to neutralize the bacteriostatic action of the sulfanilamide under our test conditions. It was found that the addition of an excess of sulfonamide-inhibitor (50 micrograms p-aminobenzoic acid) offered no apparent advantage in the initiation of growth by a smaller number of organisms, although there was some evidence to show that the aerobic organisms grow out more rapidly in the presence of an excess of p-aminobenzoic acid. The test organisms, inoculated into tubes of sulfonamide-containing Brewer's medium in numbers ranging from 3 to 100,000 grew out well in 48 hours. The assembled data on recovery of test contaminants added to Brewer's medium containing sulfanilamide is given in Table I.

TABLE I

RECOVERY OF TEST CONTAMINANTS IN BREWER'S MEDIUM* IN THE PRESENCE OF SULFANILAMIDE

TEST ORGANISM	INOCULUM NO. CELLS	SULFANILAMIDE 50 MG./TUBE		SULFANILAMIDE 50 MG. + P-AMINOBENZOIC ACID 50 γ/TUBE		CONTROL NO SULFANILAMIDE	
		24 HR.	48 HR.	24 HR.	48 HR.	24 HR.	48 HR.
ESCHERICHIA COLI	10,000	–	++	++	++	++	++
	310	–	++	++	++	++	++
	32	–	++	++	++	++	++
	3	–	++	++	++	++	++
	0	–	–	–	–	–	–
STAPHYLOCOCCUS AUREUS	100,000	–	++	+	++	+	++
	10,000	–	++	+	++	+	++
	620	–	++	–	++	+	++
	60	–	++	–	++	+	++
	6	–	++	–	++	+	++
CLOSTRIDIUM TETANI	10,000	–	++	–	++	–	++
	1,200	–	++	–	++	–	++
	224	–	++	–	++	–	++
	23	–	++	–	++	–	++
	6	–	++	–	++	–	++

*20 c.c. volumes in 25 x 200 mm. tubes. ++ = heavy growth; + = moderate growth; – = no growth.

In order to determine the efficiency of the method in the recovery of a spore-bearing pathogen from sulfanilamide powder, samples were artificially contaminated with small numbers of *Cl. tetani*. Two packages of sterile sulfanilamide powder were contaminated with 20,000 and 450 cells of *Cl. tetani*, respectively. The powders were returned to their packages, sealed and allowed to remain at room temperature for two days. Contaminated and sterile sulfanilamide powders were then examined by the test procedure. No growth was observed in the sterile sulfanilamide controls, while tubes containing contaminated sulfanilamide powder showed excellent growth of *Cl. tetani* within 48 hours. It was further noted that when the flasks containing the suspension of the artificially contaminated sulfanilamide powders were allowed to stand at room temperature for 48 hours, recovery of the organisms was effected even though the sulfonamide powder was completely dissolved and the spores of *Cl. tetani* were necessarily exposed to a 1000 mg. per cent solution of the drug for at least a portion of that length of time. This is further evidence of the extreme resistance of *Cl. tetani* spores to high concentrations of sulfanilamide.

This procedure is applicable to other sulfonamides as well. It is necessary, however, first to determine the maximum quantity of drug which is inactivated by the sulfonamide-inhibitors present in Brewer's medium.

SUMMARY

A test for the determination of sterility in sulfanilamide powder is presented. It is simple in operation, and effective in the recovery of small numbers of spore-bearing pathogens from artificially contaminated sulfanilamide samples.

REFERENCES

1. Editorial: A Death from Tetanus. An Unsterilized Container, Pharmac. J. 148: 192, 1942.
2. Long, P. H.: Sterilization of Sulfonamides, Lancet 2: 322, 1942.
3. Atkinson, E. C.: Tetanus After Sulfonamide Treatment of Cavities, Brit. M. J. 2: 24, 1942.
4. Editorial: Sterilization of Dry Powders, Brit. M. J. 2: 134, 1942.
5. Cherry, J. C.: Tetanus after Sulfonamide Treatment of Cavities, Brit. M. J. 2: 142, 1942.
6. Editorial: Tetanus After Local Chemotherapy, Lancet 1: 770, 1942.
7. Brewer, J. H.: Clear Liquid Medium for the "Aerobic" Cultivation of Anaerobes, J. A. M. A. 115: 598, 1940.
8. Landy, M., and Dicken, D. M.: A Microbiological Method for the Determination of p-Aminobenzoic Acid., J. Biol. Chem. 146: 109, 1942.

A DEVICE FOR INJECTIONS INTO THE EAR OF RABBITS*

WALTER KOCH, JERUSALEM, PALESTINE

WHEN a rabbit has to be injected again and again, difficulties arise as the principal veins become obliterated.

This difficulty can be overcome by a simple instrument. To the box (*a*) of an ordinary pocket lamp with an elevated convex lens (*b*) is attached a strong metal wire (*c*) of 4 mm. diameter, bent to correspond to the shape of the

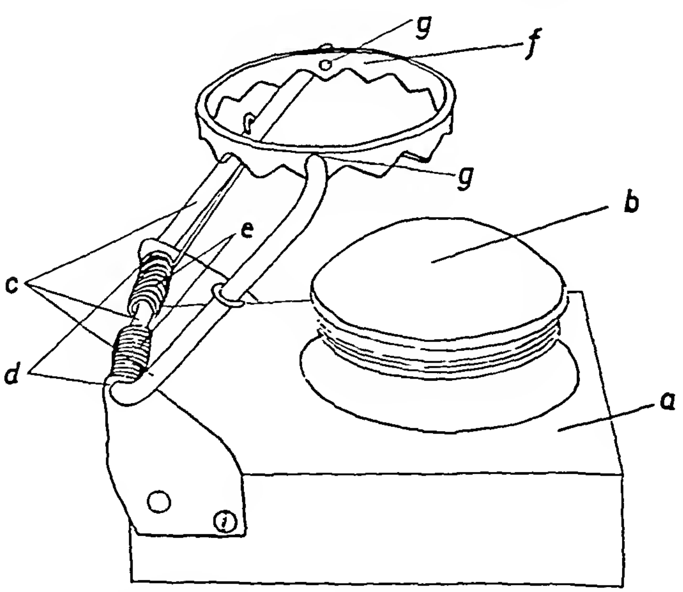


Fig. 1.—*a*, Battery casing; *b*, convex lens; *c*, metal wire; *d*, bearings; *e*, spring coils; *f*, serrated ring; and *g*, small holes in *f* into which the ends of *c* are inserted.

*From the Department of Hygiene, The Hebrew University, Jerusalem.
Received for publication, December 17, 1941.

lamp which runs in two bearings (*d*). The lower end of the wire is passed through two stiff spring coils (*e*) to give it the necessary pressure. At the upper end of the wire there is attached a serrated ring (*f*) which fits right over the convex lens of the lamp.

When using the instrument, the wire and the ring are lifted from the lens (as shown in the drawing), and the ear is interposed between lens and ring. The pressure of the springs fixes the ear with the teeth of the ring. Since the ring is serrated, the blood vessels of the ear under the teeth are compressed while there is free circulation in the vessels under the free spaces between the teeth.

The advantage of the convex lens is that it raises the ear of the rabbit above the ring so that the syringe can easily be directed to the vein to be injected without interference.

AN IMPROVED METHOD FOR DETERMINING THE SEDIMENTATION RATE OF THE RED BLOOD CORPUSCLES*

F. Rappaport, Ph.D., Palestine

According to Reichel, the sedimentation rate is independent of the height of the blood column if the depth of the sedimentation does not exceed one-third the entire blood column. Also, it is independent of the volume of the test tube if the diameter of the latter is not smaller than 1.4 mm. With a narrower tube the capillary action would interfere perceptibly with the determination. The sedimentation rate also depends on the external temperature, being accelerated by heat and retarded by cold. In order to exclude this source of error—which in tropical countries shows its effect especially in summer, while in temperate zones it is more conspicuous in the winter—the following apparatus was constructed:

Apparatus.—This consists of a wide-necked thermos flask (see Fig. 1) fitted with an inner rack to hold the sedimentation pipettes. The stand is built to hold six sedimentation pipettes, springs keeping it at a given height. At the bottom of the stand six small test tubes with side apertures (2 to 3 mm. above their lower end), are fixed by means of small springs. The sedimentation pipettes are placed into these test tubes when a determination is being carried out.

Glass tubes (see Fig. 2) with a diameter of 1.45 to 1.6 mm., approximately 21 cm. long, the lower part without graduation, and cut flat. The ungraduated part corresponds to one-fourth the graduated part, i.e., from 30 to 40 mm. The upper graduated section is divided into centimeters, showing millimeter graduation as well. Length of graduated part is from 120 mm. to 160

*From the Beilinson Hospital, Petach Tiqua, Palestine.

Received for publication, May 18, 1942.

mm. Over the top a soft and transparent tubing of Para rubber is fitted, containing a movable glass bead.

Reagents.—1. 3.8 per cent neutral sodium citrate solution (tribasic). 2. Glycerol to moisten the tubing.

Procedure.—The pipettes should be rinsed with distilled water and dried with warm air only (no acetone or alcohol). Before blood is drawn from the finger tip the entire pipette should be rinsed with citrate solution. Then the citrate should be sucked up to the ungraduated part. This citrate is blown out into a small test tube. The blood is then allowed to flow (by capillary action) into the pipette—which should lie in as horizontal a position as possible—up to mark 0. Then let the blood flow into the citrate test tube, mix it well, but avoid formation of bubbles. The sedimentation test may be set up

Fig. 1.

Fig. 2.

immediately or, at the latest, in the course of the next five hours. If the sedimentation is set up immediately, the pipette may be used again without being cleaned beforehand, otherwise it may be used only after thorough cleaning, drying, and renewed moistening with the citrate solution.

For setting up the reaction, moisten the tubing with a small drop of glycerol at its lower end. Slip almost the entire length of the rubber tubing (which is about 4 cm.) over the pipette. The glass bead pressed aside, the blood citrate mixture should be sucked up to mark 1. By drawing the rubber tubing upward, the blood column is raised up to mark 0. (If necessary

correct by shifting the bead inside the tubing.) Now the pipette is inserted into the stand which is put into the thermos flask, previously filled with water at 20° C. Readings are taken after thirty, sixty, and one hundred and twenty minutes with a slow sedimentation and after thirty and sixty minutes only if the sedimentation is fast.

REFERENCE

Reichel, H.: Blutkoerperchensenkung, Vienna, 1936, J. Springer, page 16.

A SODIUM LAURYL SULFATE SOLUBILITY TEST FOR THE IDENTIFICATION OF PNEUMOCOCCI*

MILWARD BAYLISS, PH.D., M.D., CHICAGO, ILL.

SINCE Neufeld in 1900 demonstrated that pneumococci were soluble in bile, it has been customary to differentiate this group of bacteria from streptococci by treating the organisms with bile or one of the bile salt preparations. The difficulty in obtaining a clear specimen of bile is an obvious disadvantage when it is to be used for a test in which the final criterion is the clearing of a suspension of the organisms. Within recent years sodium desoxycholate has to a considerable extent replaced the use of bile. This bile salt gives a clear colorless solution, but it has the disadvantages of being expensive and, if the pH of the media is 6.6 or less, desoxycholic acid will precipitate and give a cloudy solution which obscures the test.

Lysis or clearing is considered by some investigators to be hastened by any process that kills the pneumococcus without impairing its enzyme activity (Mair, 1929). Bile, sodium desoxycholate, sodium ricinoleate, and most of the other substances which accomplish this are surface tension depressants. Because sodium lauryl sulfate possessed this property of lowering surface tension, it was thought that it might parallel bile in its action on pneumococci and streptococci. This compound was soluble in the presence of calcium and magnesium ions and in the presence of acid or alkali. With its low cost and the ease of obtaining a clear colorless solution, it would be more satisfactory than bile or sodium desoxycholate if the same results were obtained.

Bayliss (1937) studied the visible action of sodium lauryl sulfate on various microorganisms. Gram-negative organisms were usually cleared while gram-positive organisms remained turbid when broth cultures were treated with solutions of this compound. The pneumococcus was a notable exception to this rule. All available strains of pneumococci tested were found to be cleared in thirty minutes at 37° C. when the concentration of sodium lauryl sulfate was 0.2 per cent. All streptococci tested were found to be unaffected. The ease of obtaining

*From the Department of Bacteriology and Public Health, College of Medicine, University of Illinois, Chicago.
Received for publication, May 25, 1942.

satisfactory material for carrying out this test and of reading the results, because of the water clearness of the solution of sodium lauryl sulfate, made this procedure appear to be a desirable substitute for the bile solubility test. The cultures available at that time were limited. Since then the author has continued his observations on several hundred strains of pneumococci and streptococci.

EXPERIMENTAL

The action of sodium lauryl sulfate was studied by adding 0.5 c.c. of various concentrations of crude sodium lauryl sulfate* to an equal amount of a twenty-four-hour serum infusion broth culture of the organism. Controls of the culture plus saline, as well as uninoculated media plus sodium lauryl sulfate solution were made for comparison. After thorough shaking, the tubes were examined at frequent intervals and the degree of clearing was noted.

During the past five years several hundred pneumococcus cultures, including all the 32 types for which commercial typing antisera are available, have been subjected to the action of sodium lauryl sulfate. A few strains of pneumococci which did not fall in the types for which commercial typing antisera are available have also been tested. All freshly isolated strains have been readily soluble. Occasionally an old laboratory strain was encountered which was soluble with difficulty or, in rare instances, was unaffected. In such instances the effect on the organism of sodium desoxycholate or of bile was also proportionately decreased. Treatment of the pneumococcus in a manner which prevented the lytic action of bile also stopped any effect by sodium lauryl sulfate. Cultures heated to boiling for ten minutes were unaffected by either substance.

Pneumococci which have been recently isolated showed rapid clearing even in dilutions of sodium lauryl sulfate as high as 1:2,560. Table I illustrates the comparative clearing action of this compound and of sodium desoxycholate on type XI pneumococcus.

Numerous strains of pneumococci have given results comparable to those in Table I. With these results in mind a routine test for use in the identification of pneumococci was established. This consisted of adding 0.1 c.c. of a 2 per cent solution of sodium lauryl sulfate to 0.9 c.c. of a twenty-four-hour infusion broth culture. A control in which 0.1 c.c. of normal saline was added to 0.9 c.c. of the culture was also performed, although the results were striking enough so that this was not usually necessary. Both tubes were then incubated at 37° C. for thirty minutes before a final reading was made. With this routine procedure all freshly isolated strains of pneumococci were cleared.

A 2 per cent solution was best prepared by making 2 Gm. of crude sodium lauryl sulfate up to 100 c.c. with water. It was found desirable to heat, but

*"Dreft" is a sodium lauryl sulfate product manufactured by Procter and Gamble Company. It consists of about 50 per cent inorganic material, mainly sodium sulfate, and about 50 per cent organic compounds. Of the latter, about half is sodium lauryl sulfate and the remainder are compounds very similar in structure and properties, such as sodium sulfate esters of decyl, myristyl, cetyl, and oleyl alcohols. The normal variation of composition of the organic substances corresponds to that of coconut oil and palm kernel oil from natural sources.

"Dreft" is very soluble in water but frequently on standing at room temperature a white precipitate will settle out of the solution. This can easily be redissolved by warming. If kept at 37° C., the solution remains clear indefinitely.

McClure and Harris (1940) have recommended the use of a similar sodium lauryl sulfate product, Duponol WA, in solubility tests for pneumococci.

TABLE I

COMPARISON OF THE CLEARING ACTION OF SODIUM LAURYL SULFATE AND SODIUM DESOXYCHOLATE ON DIPLOCOCCUS PNEUMONIAE (TYPE XI)

CONCENTRATION	SODIUM LAURYL SULFATE					SODIUM DESOXYCHOLATE				
	TIME (MINUTES)					TIME (MINUTES)				
	1	3	5	10	30	1	3	5	10	30
1:20	1	2	3	4	4	2	3	4	4	4
1:40	1	2	3	4	4	2	3	4	4	4
1:80	1	2	4	4	4	2	2	4	4	4
1:160	2	3	4	4	4	2	2	4	4	4
1:320	2	3	4	4	4	2	2	3	4	4
1:640	2	3	4	4	4	2	2	2	3	4
1:1,280	1	2	4	4	4	-	1	2	2	3
1:2,560	1	2	3	4	4	-	-	-	1	2
1:5,120	-	1	1	2	3	-	-	-	-	1
1:10,240	-	-	-	-	1	-	-	-	-	-
1:20,480	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-

Key:
4, 90-100 per cent clearing.
3, 50-90 per cent clearing.
2, 25-50 per cent clearing.
1, 10-25 per cent clearing.
-, less than 10 per cent clearing.

not to autoclave, to aid in dissolving the compound. To remove any particles of dirt it was passed through filter paper. When kept at 37° C., it remained ready for use at any time.

Numerous strains of streptococci, including alpha, beta, and gamma types have been unaffected by the sodium lauryl sulfate solution. In each instance there has been either no clearing action or occasionally a slightly increased turbidity, possibly due to the increased dispersion of the intact organisms by this surface tension depressant. Parallel tests with bile and sodium desoxycholate have in each instance given similar results.

Microscopic examination at frequent intervals of the pneumococcus cultures following the addition of the sodium lauryl sulfate demonstrated decreasing numbers of intact organisms. With streptococci no disappearance of the bacteria was noted. Cultures of gram-negative bacteria to which relatively high concentrations (1:50 to 1:100) of the compound have been added usually showed macroscopic clearing, with a marked increase in the viscosity of the media, though microscopic examination revealed no marked lytic action on gram-negative organisms, such as *Escherichia coli*.

SUMMARY

Sodium lauryl sulfate rapidly cleared suspensions of pneumococci with disintegration of the organisms. Several hundred strains of pneumococci of various types have been studied and found to be lysed rapidly in a 0.2 per cent concentration of the compound.

All freshly isolated strains showed definite clearing within thirty minutes. Streptococci under the same conditions were unaffected.

The test is best performed by adding 0.1 c.c. of a 2 per cent solution of crude sodium lauryl sulfate ("Dreft") to 0.9 c.c. of a 24-hour culture. Usually

the results can be determined in a few minutes, but if clearing does not occur, it is necessary to incubate the mixture at 37° C. for one-half hour before the final reading.

REFERENCES

Neufeld, F.: Ueber eine specifische bakterolytische Wirkung der Galle, Ztschr. f. Hyg. u. Infektionskr. 34: 454, 1900.
Mair, W.: Autolysis and Solubility of Pneumococcus in Bile, System of Bacteriology in Relation to Medicine, Med. Res. Council Great Britain, London, H. M. Stationery Office 2: 168, 1929.
Bayliss, M.: Visible Action of Sodium Lauryl Sulfate on Microorganisms, J. Lab. & Clin. Med. 22: 700, 1937.
McClure, G. Y., and Harris, A. H.: The Use of a Detergent in Solubility Tests for the Identification of Pneumococci, Annual Report of the Division of Laboratories and Research of New York State Health Department, page 47, 1940.

A METHOD FOR MAKING SECTIONS OF BONES AND TEETH*

Elbert B. Ruth, A.M., Ph.D., Cincinnati, Ohio

THE usual methods of studying the finer aspects of bone and tooth structure employ thin celloidin or paraffin sections, or slabs of bones or teeth sawed from the specimen and ground as thin as possible by some appropriate technique. The former method yields satisfactory studies of the cytologic picture, and the latter is capable of producing excellent studies of Haversian systems with their lamellae, lacunae, and canaliculi. Both of these methods are valuable and necessary, but both have certain drawbacks.

The method herein described, in supplementing the other methods, gives excellent pictures of bone architecture, with only the canaliculi showing poorly. The lamellae and lacunae, however, are demonstrated in a striking manner not seen in ground sections, or in the celloidin or paraffin sections. An additional advantage is obtained through the use of unstained and relatively thick (80μ to 120μ) sections, which offer a three-dimensional picture.

PROCEDURE

This method may be applied to bone in any state—fresh, fixed, or ordinary dried skeletal material. The best results are obtained when the bone is free from soft tissues and grease. Fresh bone may be fixed first or macerated in warm water until clean. Any appropriate fat solvent may be used for degreasing; acetone works well with fresh bone, and either acetone, benzene, or gasoline is satisfactory for dry bones.

Decalcification is accomplished in 5 per cent hydrochloric acid. The use of nitric acid is not recommended, since it seems to make the organic matrix brittle in some cases, and this results in a certain amount of splitting of the tissue. Hydrochloric acid decalcifies rapidly and does not cause maceration of the organic matrix if the tissue is not left in the reagent too long. Decalcification is considered to be complete when about 10 c.c. of the acid solu-

*From the Department of Anatomy, College of Medicine, the University of Cincinnati.
Received for publication, May 25, 1942.

tion covering the specimen is added to an equal volume of equal parts of 5 per cent ammonium hydroxide and 5 per cent ammonium oxalate, and no precipitate is formed. When decalcification is complete, the tissue is washed in tap water until the acid is removed.

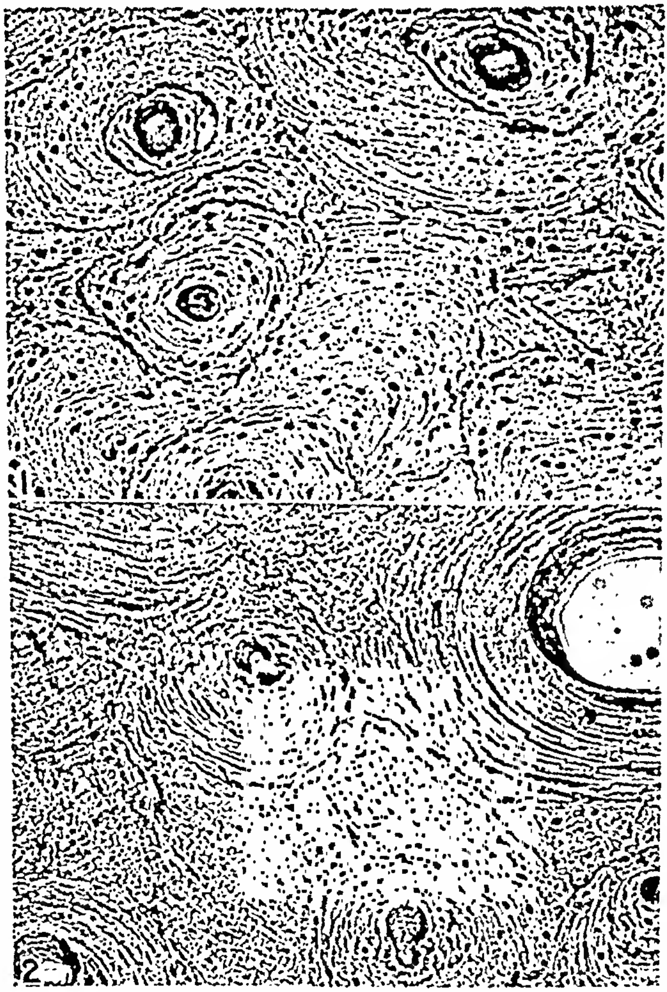


Fig. 1.—Section of a human metacarpal bone viewed with the substage condenser lowered and the concave mirror tilted to bring out the lamellar structure. The black dots are the lacunae (×160).

Fig. 2.—Another field of the same section as Fig. 1, showing the third dimensional effect obtained when focusing on a greatly enlarged Haversian canal. This section was cut freehand with a razor blade (×160).

The tissue next is placed in a potassium hydroxide-glycerin solution, such as is used in the Schultz technique for clearing embryos for demonstration of the skeleton. The formula used is:

Water	77 c.c.
Glycerin	20 c.c.
Potassium hydroxide	3 Gm.

The tissue remains in this solution until the matrix becomes semitransparent, when it is placed in rising grades of glycerin (50 per cent, 75 per cent, and 100 per cent) until completely cleared.

The glycerin is removed from the tissue by washing in several changes of 70 per cent alcohol. This is followed by replacing the 70 per cent ethyl alcohol with n-butyl alcohol, C.P. Two or three baths of butyl alcohol may be required in order that there may be no ethyl alcohol left in the tissue. The last butyl alcohol bath may be saved, and the alcohol may be used later to dissolve the paraffin out of the cut sections.

The next step is to infiltrate the specimen with paraffin and to embed for sectioning. At least three paraffin baths (one hour or longer in each bath—according to the size of the specimen) are recommended.

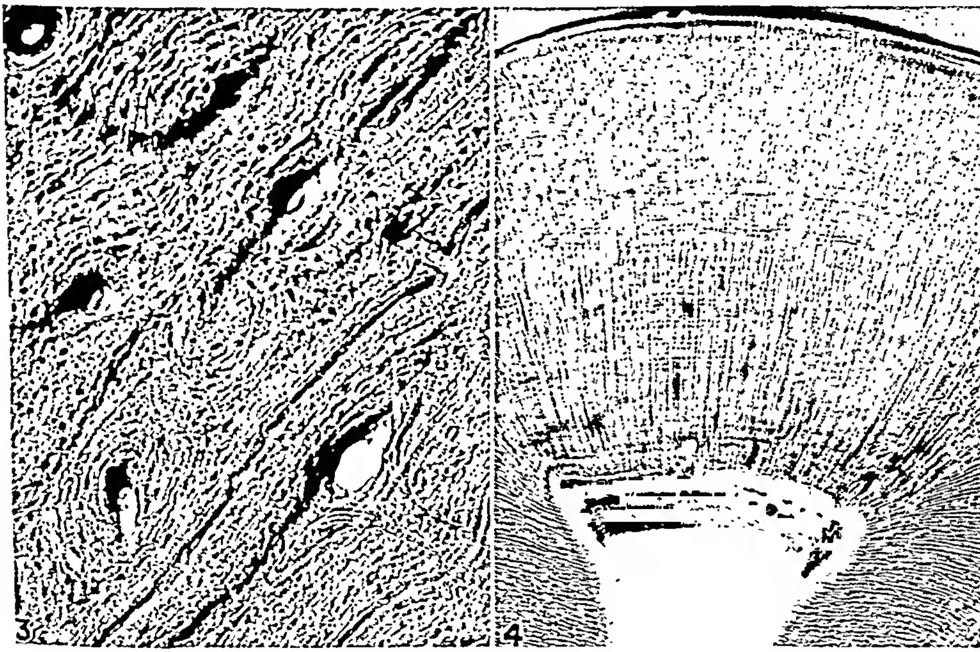


Fig. 3.—Section of the femur of a child about 8 years old. The substage condenser has been elevated as high as possible, and the concave mirror is tilted to give the impression of viewing the section by reflected light. Vascular channels are shown well, and the third dimensional effect is demonstrated. The black dots are lacunae. Section cut on microtome—120μ ($\times 160$).

Fig. 4.—Section of the tooth of a fish to illustrate the application of this method to the sectioning of teeth. Freehand section cut with a razor blade ($\times 160$).

Sections may be cut in any of three ways: (1) freehand with a razor blade; (2) with a well microtome and a straight-edge razor, or a microtome knife; or (3) with a celloidin microtome that will cut sections up to 200μ. The sections in Figs. 1, 2, and 4 were cut freehand. The section in Fig. 3 was cut at 120μ on a celloidin microtome. By cutting sections of different thicknesses, from 40μ to 200μ it appears that the sections from 80μ to 120μ give the best pictures of the arrangement of the circumferential lamellae, Haversian systems, interstitial lamellae, and the interrelationships of the Haversian canals.

Sections are put into a container with enough butyl alcohol to cover them. They remain rolled up until the butyl alcohol has dissolved away the paraffin in which they are embedded. Removal of the paraffin is accomplished

in the paraffin oven. The first butyl alcohol bath will remove most of the paraffin, but two more baths in butyl alcohol are necessary to remove all traces of paraffin. Glycerin is added to the butyl alcohol of the final bath in rising percentages—25 per cent, 50 per cent, and finally 100 per cent. Glycerin replaces the butyl alcohol and clears the sections.

The sections are mounted more or less permanently in glycerin jelly. They may be studied in glycerin, but need to be in a semisolid medium for photomicrography, or for handling over a period of time. Greater permanence may be given the slides by ringing the cover slip with gold size and Bell's cement. A thick jelly is recommended, according to the following formula:

U.S.P. granular gelatin	15 Gm.
Distilled water	50 c.c.
Glycerin	55 c.c.
Phenol crystals	2 Gm.

The gelatin is put into the water and melted with gentle heat. The glycerin may be added at this time after first dissolving the phenol in the glycerin. The melted glycerin jelly should be filtered through moist, hot flannel before using.

The sections are taken from pure glycerin and pressed between two filter papers to remove excess glycerin. The melted jelly is pipetted onto a warmed slide (the number of drops necessary is easily ascertained after several trials), and the section is placed in the jelly. The coverslip is applied, after warming over a flame, and the excess gelatin is pressed out from under the cover slip. Slides are readily cleaned after the gelatin hardens by cutting away any gelatin extending onto the slide beyond the cover slip. Wiping the slide with a damp cloth completes the cleaning process.

Proper use of the microscope in the study of the prepared slides is quite as important as the different steps of preparation of the tissue. The lighting effects that are desired to bring out the details of the sections, are obtained by manipulation of the substage condenser and mirror. In one position the section appears to be illuminated by reflected light, as seen in Fig. 3. Another position of the mirror and condenser gives the appearance of lighting by transmitted light, as in Figs. 1, 2, and 4. In the former position the surface topography of the section is seen in three dimensions, and by proper focus the details of the vascular channels and lacunae are emphasized. These details may be studied with various magnifications. In the latter position the details of lacunae and lamellae are emphasized. This brings out the true architecture of the bone—the boundaries of the Haversian systems and the relations of the interstitial and circumferential lamellae being displayed in a striking manner. To produce the effect of reflected light the condenser is raised as high as possible, and the mirror is tipped in different directions until the desired result is obtained. To bring out the lamellar structure the condenser is slowly lowered, and again the mirror is moved in different directions until the desired effect is obtained. The concave mirror is always used.

The use of the higher grades of ethyl alcohol for dehydration, and xylol, or other clearing reagents ordinarily used before passing the tissue into

paraffin, hardens the ostein, and changes it optically so that the structural details are shown very poorly, or not at all.

The use of Lendrum's technique (Carleton, 1938) for softening dense fibrous tissue was found to be advantageous with teeth and with bones larger than rat bones. The decalcified bone is placed in a solution of 4 per cent phenol for four to six days at room temperature. If any signs of maceration appear, the treatment must be stopped at once. The bone is washed in several changes of water at 45° C. until the odor of phenol no longer can be detected. The specimen is then cleared as described. The rest of the treatment is unchanged.

SUMMARY

Fresh, fixed, or dried bone specimens are decalcified in 5 per cent hydrochloric acid.

The decalcified bone is cleared in potassium hydroxide-glycerin solution, and finally in rising grades of glycerin (50 per cent, 75 per cent, and 100 per cent).

Glycerin is removed from the tissue by washing in 70 per cent alcohol, which is replaced by n-butyl alcohol (three baths).

The specimen is infiltrated with paraffin* and is embedded in a block of suitable size. Sections may be cut freehand with a razor blade; on a well microtome, with a section razor or microtome knife; or on a celloidin microtome. Thick sections are used for study, 80μ to 100μ.

Paraffin is dissolved out of the sections in several changes of n-butyl alcohol, and the sections are placed in several changes of rising grades of glycerin: 25 per cent, 50 per cent, and 100 per cent.

From 100 per cent glycerin the sections are mounted in a thick glycerin jelly, and are studied microscopically with different lighting effects attained by the raising and lowering of the substage condenser, and the movement of the concave mirror to reflect the light from different angles.

REFERENCE

Carleton, H. M.: Histological Technique, New York, 1938, Oxford University Press, page 39.

*Parowax produced by the Standard Oil Company of Ohio, Cleveland, Ohio, is used in this laboratory.

EYEGLASSES TO BE USED WITH THE BINOCULAR MICROSCOPE*

Alfred Plaut, M.D., New York, N. Y.

FOR several years a pair of special eyeglasses has proved helpful to me, a hyperopic person, while using the binocular microscope. The glasses are crescent-shaped, 1.3 cm. high in the center. Over the upper concave border, I look into the oculars of the microscope. With the bitukni binocular one can sit in a very convenient position without strain on the muscles of the neck. (These eyepieces form an angle of 135° with the vertical tube.) I had about one-third of the upper rim of the oculars removed on the lathe, flush with the tube of the eyepiece, enabling me to get as near the eyepieces as can be done without glasses, and thus getting the benefit of the whole microscopic field. It also prevents the eyeglasses from catching at the microscope and it permits free movement of the head. When the upper portion of the eyepieces is cone-shaped, as in the Spencer outfit, this is unnecessary.

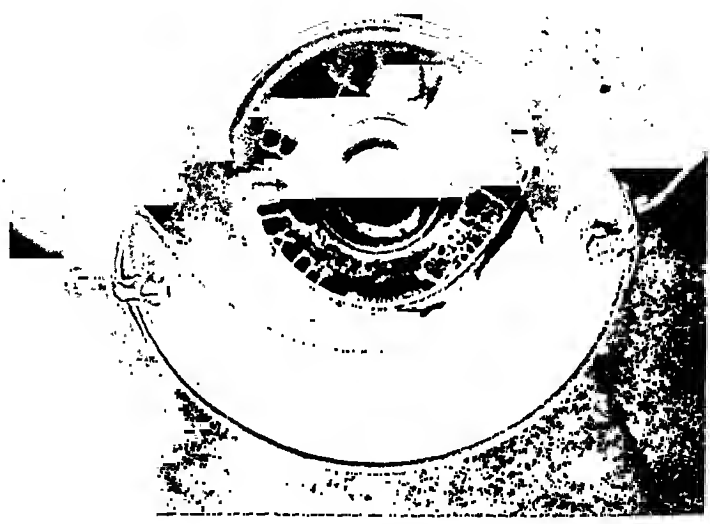


Fig. 1.

The glasses are weaker than the ones used for reading. Hence, without eye-strain and without much moving of the head, it becomes possible to overlook the table I am working on. At the same time, the glasses are strong enough for writing and reading. The strength of the glasses is a matter of individual choice. Except for continued reading and for observation of finer details, such glasses can be used for most laboratory activities. This obviates the too frequent changing of eyeglasses. Fig. 1 is self-explanatory.

*From Beth Israel Hospital, New York City.
Received for publication, June 1, 1942.

THE PITUITRIN CONCENTRATION TEST OF RENAL FUNCTION*

NORMAN G. SCHNEEBERG, M.D., WILLIAM B. LIKOFF, M.D., AND
I. EDWARD RUBIN, M.D., PHILADELPHIA, PA.

POSTERIOR pituitary extract inhibits water diuresis by producing an increased reabsorption of water by the renal tubular epithelium. Its use, therefore, has been advocated as a substitute for the required period of water deprivation necessary in the performance of the standard Fishberg test. This period of water restriction represents a considerable inconvenience to the patient and a source of error when the patient fails to cooperate.

The use of pituitary preparations in the performance of kidney function tests was first advocated in 1930,[1] and since then sporadic references to its use have appeared in the literature.[2] Recently, Sodeman and Engelhardt[3] attested to the efficacy of surgical pituitrin in producing a urine, in 15 normal subjects, as concentrated as that produced by a "modified Fishberg technique."

The purpose of this study is (1) to compare, in unselected hospital cases, the urine concentrations obtained after an injection of surgical pituitrin without a period of water deprivation, with urine concentrations obtained in the same patient following the usual period of water restriction advocated by Fishberg; and (2) to judge the clinical applicability of the pituitrin test.

METHODS

Four procedures were employed:

1. Fishberg concentration test: The technique used was identical with that outlined by Fishberg.[4]
2. Pituitrin concentration test: Without fluid or food restriction the patient emptied his bladder at some designated hour, and 0.5 c.c. of surgical pituitrin (10 units) was injected subcutaneously. Three urine specimens were obtained hourly thereafter.
3. Volhard dilution test: 1,500 c.c. of fluid were administered orally within thirty minutes after emptying the bladder. Four hourly urine specimens were then collected.
4. Procedure 3 was repeated, but in addition 0.5 c.c. of surgical pituitrin (10 units) was injected after the ingestion of the fluid.

The specific gravity of the urine specimens obtained was measured by means of a clinical urinometer calibrated to a standard temperature. Corrections were made for temperature variations[5] and protein content[6] of the urine.

In 100 patients procedures 1 and 2 were carried out. In 25 of these all four procedures were performed.

For the purposes of this study a specific gravity of 1.020 was arbitrarily chosen to divide the cases into two groups. Those patients concentrating to 1.020

*From the Laboratory of the Mount Sinai Hospital, Philadelphia.
Received for publication, June 24, 1942.

or better in any single specimen were considered to have normal renal function, while those not reaching that standard were grouped as representing abnormal renal function. The pitnitrin test was considered to duplicate the Fishberg test when it succeeded in producing a urine which fell into the same group classification as that obtained with the latter.

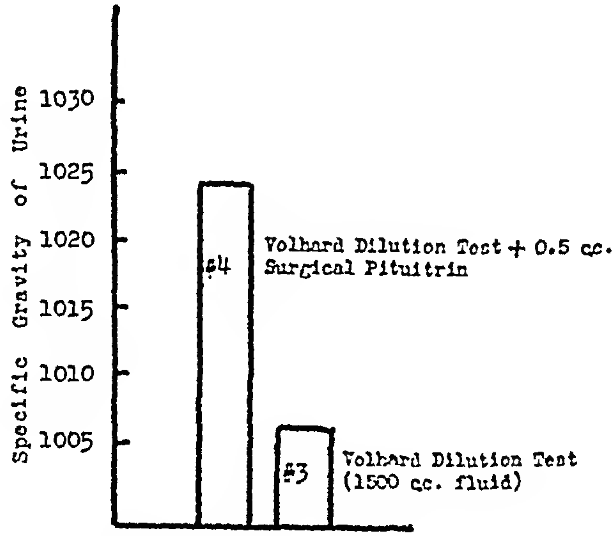


Fig. 1.—Average urine concentration produced by procedures 3 and 4.

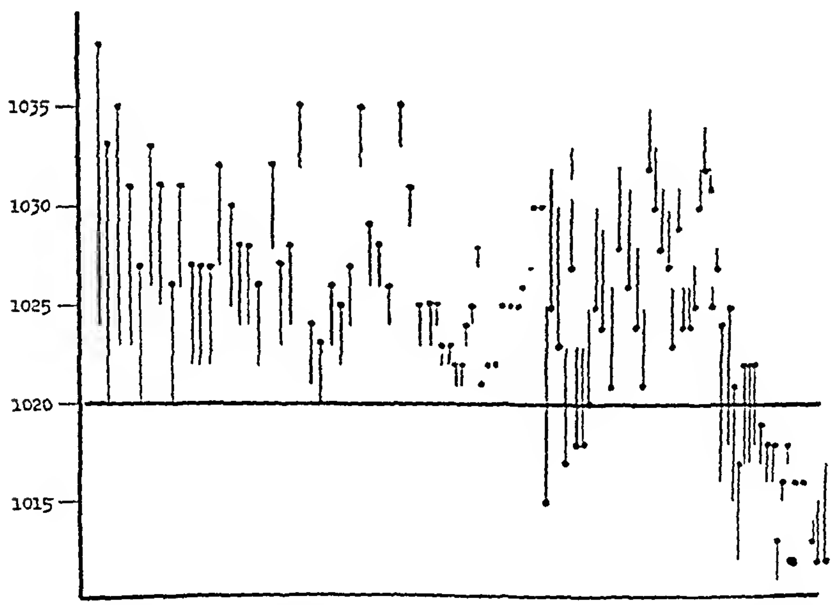


Fig. 2.—A comparison of Fishberg and pituitrin concentration tests of renal function in 100 patients.
• Pituitrin test (procedure[2])—highest concentration.
| Fishberg test (procedure[1])—highest concentration.

RESULTS

In 25 patients the Volhard dilution test (procedure 3) produced a dilute urine in contrast to the definitely more concentrated urine obtained when this

test was combined with the use of pituitrin (procedure 4). The average concentrations obtained are graphically reproduced in Fig. 1.

The pituitrin test compared favorably with the Fishberg test in 90 of 100 cases.

Of 81 patients, comprising a group concentrating to 1.020 or above by means of the Fishberg technique, 4 (4.9 per cent) failed to reach that specific gravity when the pituitrin technique was substituted. Of the 19 patients comprising the group with urine concentrations below 1.020 with the Fishberg technique, 6 (31.5 per cent) showed concentrations over 1.020 with the pituitrin test. These results are graphically represented in Fig. 2.

No deduction can be drawn as to the effect of pituitrin on urinary volume. While on the whole more concentrated urines were accompanied by a reduction in volume, examples were encountered in which alterations in specific gravity were independent of urinary volume.

DISCUSSION

The production of concentrated urines after the injection of 0.5 c.c. (10 units) of surgical pituitrin in the face of a quantity of ingested fluid that ordinarily would have produced a very dilute urine (procedure 3 and 4) definitely established the potency of the preparation of pituitrin used in these experiments.

It is apparent from the results obtained that the pituitrin test duplicated the Fishberg in 90 per cent of the cases investigated.

Of the 10 cases representing failure of correlation of the two tests, in only four did the pituitrin test fail to reach the high concentrations obtained with the Fishberg test. In the remaining six patients the pituitrin technique produced a sufficiently more concentrated urine to alter the estimation of kidney function from abnormal to normal. A clinical and laboratory evaluation of these six cases substantiated the interpretation derived from the pituitrin technique in that there was no history to suggest a latent nephritis, blood pressures were all within normal range, repeated urinalysis revealed no abnormal constituents, and the blood urea clearance tests in each instance were above 80 per cent of normal.

No untoward effects have been observed with the use of pituitrin even in severe hypertensives and in patients with coronary artery disease. We have avoided performing the test in pregnancy.

The technique of the test is simple, causes a minimum of inconvenience to the patient, and by eliminating the need for any specific preparation, permits the physician to exercise more complete control over the patient.

A statistical analysis of the 100 cases* revealed an average difference in specific gravity between the pituitrin and Fishberg tests of -0.001, with a standard deviation of 0.0043. Thus, in any given instance within the statistical limits of ±2 times the standard deviation, the pituitrin test may yield a specific gravity of 0.009 more than, or 0.007 less than, the Fishberg test.

Since the normal kidney can produce specific gravities of greater variation than the abnormal, the cases falling below 1.020 were analyzed separately. These yielded an average difference of +0.002, with a standard deviation of 0.0033.

*Martin S. Abel, M.D.

Thus, in any given instance within the statistical limits of ±2 times the standard deviation, the pituitrin test may yield a specific gravity of 0.008 more than, or 0.004 less than, the Fishberg.

Of the 100 cases, 47 had specific gravities ranging from 1.015 to 1.025. This significant intermediate zone revealed an average difference of −0.0002 and a standard deviation of 0.0029. Thus, values with the pituitrin test can vary from 0.005 more than, or 0.005 less than, the Fishberg in this group.

The analysis further indicates that any given pituitrin test yielding values of 1.025 or above, or 1.015 and below, will almost invariably agree with the Fishberg test. Within the range of 1.015 to 1.025 the probability of the two tests agreeing becomes less, diminishing as the arbitrary dividing line of 1.020 is approached.

However, the analysis presupposes that the Fishberg test is a constant standard and will not vary appreciably from day to day. We demonstrated this basic supposition to be invalid by a survey of 12 patients who revealed a significant day to day variation in specific gravities obtained with the Fishberg technique.

Before the pituitrin test may be accepted as a substitute for the Fishberg on the basis of its technical advantages and 90 per cent correlation revealed in this series, or rejected on the basis of its statistical probability of disagreement with the Fishberg test, one must substitute more substantial criteria for judging the clinical applicability of this new test. This can only come about in the future through long-range observation of patients in whom both tests have been performed.

SUMMARY

1. Surgical pituitrin in the dose used (0.5 c.c. or 10 units) is an active preparation capable of concentrating urine.

2. The substitution of pituitrin for the period of water deprivation used in the Fishberg test resulted in a 90 per cent correlation of the two tests.

3. Absolute failure of the test was encountered in only four cases.

4. In six cases the pituitrin test apparently more accurately reflected kidney function than the Fishberg test.

5. Statistical analysis of the results revealed an appreciable possibility of disagreement between the two tests when specific gravities range from 1.015 to 1.025.

6. A true clinical evaluation of the test will depend upon further comparative studies.

REFERENCES

1. Leberman, F.: Funktionelle Nierendiagnostik durch Hypophysenpräparate, Med. Welt. 4: 1144, 1930.
2. (a) Corelli, F., and Bartoloni, M.: Ricerche sull'influenze dell'ormone retroipofisario sulla prova della concentrazione, Policlinico (sez. med.) 44: 382, 1937.
(b) Goldemberg, L.: Exploración de la adaptabilidad o elasticidad funcional del riñón por la prueba de Volhard combinada con la inyección de retropituitrina, Dia méd. 3: 756, 1931.
Goldemberg, L.: Exploración de la adaptabilidad o elasticidad funcional del riñón por la prueba de Volhard combinada con la inyección de retropituitrina, Prensa méd. argent. 17: 1708, 1931.

(c) Marcolongo, F.: L'influenze dell'ormone retroipofisario sulla funzione renale nelle nefropatie sotto l'aspetto clinico e prognostico, Minerva med. 2: 106, 1935.
(d) Pasqualini, R. Q., and Etala, E.: Determinación de la capacidad de reabsorción del tubulo renal por medio de extractos de lóbulo posterior de hipófisis (reabsorción tubular forzada), Rev. Soc. argent. de biol. 16: 161, 1940.
(e) Silvette, H.: Influence of Post-pituitary Extract on Excretion of Water and Chlorides by Renal Tubules, Am. J. Physiol. 128: 747, 1940.
3. Sodeman, W. A., and Englehardt, H. T.: Renal Concentration Test Employing Use of Pituitary Extracts. Response of Normal Subjects, Proc. Soc. Exper. Biol. & Med. 46: 688, 1941.
4. Fishberg, A. M.: Hypertension and Nephritis, ed. 3, Philadelphia, 1934, Lea & Febiger, page 58.
5. Gradwohl, R. B. H.: Clinical Laboratory Methods and Diagnosis, St. Louis, 1935, C. V. Mosby Co., page 50.
6. Lashmet, F. H., and Newburgh, L. H.: Improved Concentration Test of Renal Function; Simple Method for Measuring Proteinuria, J. A. M. A. 100: 1328, 1933.

CHEMICAL

THE STANDARDIZATION AND ASSAY OF HEPARIN BY THE TOLUIDINE BLUE AND AZURE A REACTIONS*

Alfred L. Copley, M.D.,† New York, N. Y., and David V. Whitney III, B.S., Chem. E., Kansas City, Mo.

We believe that in view of the increasing use of heparin a direct chemical assay method is of decided advantage. In two previous papers[1, 2] the toluidine blue method of heparin assay (method I) was partially outlined. The present paper is a result of our efforts to increase the accuracy and to widen the scope of this method. In addition, since the biological assay methods for heparin differ greatly in results,[3] it seemed advisable to attempt the standardization of the unit of heparin by employing the colorimetric assay.

The toluidine blue reaction was described by Lison[4, 5] as specific only for sulfuric acid esters of high molecular weight, referring primarily to chondroitin sulfuric acid. Chondroitin sulfuric acid has the amino sugar galactosamine, whereas Jorpes and Bergstroem[6] demonstrated that heparin contains glucosamine. They identified heparin as a mucoitin polysulfuric ester and Jorpes[7] tested it with toluidine blue. Heparin was found to be a mucoitin sulfuric acid residue by Charles and Todd,[8] and these groupings were shown in the analytical values of the sodium salt by Reinert and Winterstein.[9] Barium heparin was shown by Charles and Todd[8] in a recent communication to vary in potency with the sulfur content.

Toluidine blue is not the only dye which gives a metachromatic reaction with sulfuric acid esters, for Conn[10] has shown that toluidine blue is very similar in composition to azure A. Therefore, we undertook to test azure A with regard to its relationship to heparin and in comparison with toluidine blue.

EXPERIMENTAL

We have used the following dyes manufactured by the National Aniline Chemical Company, toluidine blue under the name NU-3, and azure A under the name of NAz-7,‡ as certified by the Commission on Standardization of Biological Stains, Agricultural Experiment Station, Geneva, N. Y. Both products are on the market and, as Dr. Conn has been advised by the manufacturer, they have on hand enough NU-3 recently manufactured to last for about four years.§

*From the Hixon Laboratory for Medical Research, University of Kansas School of Medicine, Kansas City.

Received for publication, April 6, 1942.

†Goldwater Memorial Hospital, Welfare Island, New York.

‡We are indebted to Dr. H. J. Conn for rechecking our sample NU-3 and for supplying us with the sample of Azure A (NAz-7).

§Personal communication, October 29, 1941.

We have been supplied with preparations of heparin by Connaught Laboratories, Lederle Laboratories, and Abbott Laboratories which were assayed biologically by Dr. A. F. Charles, Dr. C. M. Meadows, and Dr. W. C. Risser, respectively.

METHOD I

Procedure.—Make up 1:1,000 toluidine blue (TB) or azure A (AA) by adding 1.6949 Gm. TB (NU-3) to 1,000 c.c. or 1.1494 Gm. AA (NAz-7) to 1,000 c.c. of distilled water. In a series of test tubes place 1 u (Murray-Best unit*), 2 u, 3 u, 4 u, and 5 u of heparin CLT-110 (Connaught Laboratories, Toronto. 1 mg. containing 110 u), make up to 5 c.c. with distilled water, and add 1 c.c. of 1:1,000 toluidine blue (TB). 100 per cent dye strength. Shake, and after standing for fifteen minutes, centrifuge at 3,600 r.p.m. for fifteen minutes. The supernatant fluid is decanted and compared on the Duboscq colorimeter with a solution of 1:6,000 toluidine blue (100 per cent dye strength). The concentrations of dye are computed in gamma. Six cubic centimeters of the 1:6,000 toluidine blue solution contain 1,000 gamma of dye. It has been discovered by experience that those solutions containing from 300 gamma to 700 gamma give the most accurate comparisons. In view of this experience we select those concentrations between these limits for our computations and take the average as our result. After the residual dye concentrations have been computed, they are each substracted from 1,000 gamma (the original dye concentration), giving the amount of dye reacting with the heparin present. Dividing this quantity by the units of heparin in the test, the amount of dye reacting with each unit of heparin is obtained. Dividing the amount of dye reacting with each unit by 150 gives the strength in colorimetric (Copley-Whitney) units.

Results.—In the test on a Connaught Laboratories sample, 110 u per milligram (CLT-110), that we used as a standard, the following concentrations were determined: The 3 u test contained 585 gamma of dye and the 4 u test contained 431 gamma of dye after centrifugation. Subtracting these amounts from 1,000 gamma, the initial concentration, we obtain 415 gamma and 569 gamma, respectively, as the amounts of dye that reacted with 3 u and 4 u of heparin. Therefore, 415 gamma divided by 3 gives 138 gamma of dye reacting with each unit of heparin, and 569 gamma divided by 4 gives 142 gamma of dye reacting with each unit of heparin. The average of 138 gamma and 142 gamma is 140 gamma per unit of heparin. Since the valuation of the Connaught Laboratories appeared to us rather high, and in order to simplify future calculations and standardization, we decided to establish 150 gamma of dye per unit of heparin as the standard. Samples were diluted up to 1 u (Copley-Whitney) per cubic centimeter and tested as in the preceding method.

Three and 4 colorimetric unit tests diluted 1:5 and 1:10 were compared on the K & E spectrophotometer (Fig. 1). Samples of TB 1:10,909 and 1:15,000, diluted 1:5 and 1:10 and compared on the K & E spectrophotometer, were found to approximate the 3 unit and 4 unit tests, respectively. From the above comparisons, 0.15 mg. of 100 per cent TB—TB (NU-3) 59 per cent dye strength as TB; AA (NAz-7) 87 per cent dye strength as TB—were

*Murray and Best[11] fixed the unit of heparin as 0.01 mg. of a crystalline barium salt. The unit of the "pure" sodium heparin, which is based upon the barium salt, equals 1/110 mg.

absorbed by each 1 u of heparin. On comparing subsequent tests on dilute solutions of heparin with standard TB and AA solutions of 1:10,909 = 3 u and 1:15,000 = 4 u in the Duboscq colorimeter, the accuracy was within less than 1 u of heparin provided the heparin solution as stock sample contained less than 30 u per cubic centimeter.

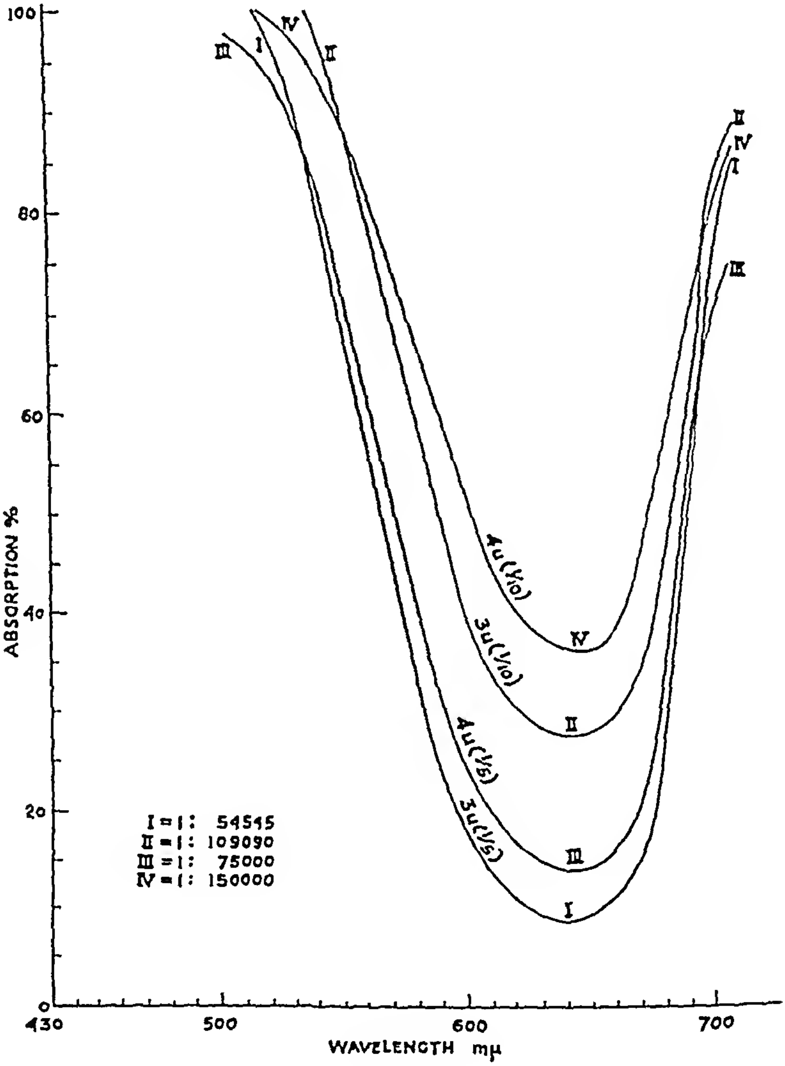


FIG. 1.

In Table I various lots of heparin from different sources were run at the indicated dilutions. These dilutions were calculated to make a solution containing about 1 u per cubic centimeter. The concentrations listed are the averages of three tests each, no concentration varying more than 50 gamma from the average. To arrive at the units per cubic centimeter we subtract the residual concentrations of TB in 3 u and 4 u tests from the original concentration of 1,000 gamma, then divide each by the decolorization value of 1 u or 150 gamma, then divide by 3 u and 4 u, respectively, and average the results. For example, taking

sample No. 1: 3 u = 585 gamma, 4 u = 431 gamma: 1,000 gamma − 585 gamma = 415 gamma, 1,000 gamma − 431 gamma = 569 gamma; 415 gamma ÷ 150 = 2.76 u, 569 gamma ÷ 150 = 3.79 u: 2.76 u ÷ 3 = 0.92 u, 3.79 u ÷ 4 = 0.95 u; 0.92 u and 0.95 u averages 0.935 u.

TABLE I

THE COLORIMETRIC UNIT OF HEPARIN WITH THE DUBOSCQ COLORIMETER AS EXPRESSED BY THE DECOLORIZATION OF A TOLUIDINE BLUE SOLUTION OF 1,000 GAMMA PER 6 C.C. FOLLOWING THE ADDITION OF 1 TO 5 ANTICOAGULANT UNITS OF HEPARIN

NUMBER	PRODUCER	DILUTION	CONCENTRATION OF TOLUIDINE BLUE, γ						U/C.C.	ORIGINAL STRENGTH
			1 U	2 U	3 U	4 U	5 U	CONTROL		
1	CLT	1 mg./110 c.c.	856	713	585	431	341	1,000	0.94	103 u/mg.
2	CLT	1 mg./ 15 c.c.	778	589	385	243	0	1,000	1.31	20 u/mg.
3	CLT	1 mg./110 c.c.	848	663	552	443	326	1,000	0.96	106 u/mg.
4	HLR	1 c.c./400 c.c.	718	519	342	211	0	1,000	1.38	552 u/c.c.
5	HLR	1 c.c./400 c.c.	777	571	397	261	0	1,000	1.28	512 u/c.c.
6	LL	1 c.c./1,100 c.c.	774	641	473	410	298	1,000	1.06	1,166 u/c.c.
7	LL	1 mg./100 c.c.	929	659	505	454	315	1,000	0.99	99 u/mg.
8	LL	1 mg./100 c.c.	776	623	534	493	396	1,000	0.93	93 u/mg.

CLT = Connaught Laboratories; HLR = Hoffmann-LaRoche; LL = Lederle Laboratories.

TABLE II

PHOTOELECTRIC (EVELYN) STUDIES WITH 3 AND 4 COLORIMETRIC UNIT TESTS OF TOLUIDINE BLUE (TB) AND AZURE A (AA)

FILTER NUMBER Mμ	THREE UNITS			FOUR UNITS		
	TB	AA	AVERAGE	TB	AA	AVERAGE
490	60.5	67.5	64.0	67.8	71.8	69.8
520	42.3	48.0	45.2	52.0	57.0	54.5
540	21.0	23.0	22.0	32.0	32.8	32.4
635	0.5	1.5	1.0	1.0	3.8	2.4
660	8.0	15.0	11.5	9.0	18.0	13.5

Center setting = 75. Each value represents average of duplicate tests.

TABLE III

COMPARISON BETWEEN VARIOUS DILUTIONS OF AZURE A OR TOLUIDINE BLUE ON THE EVELYN PHOTOELECTRIC COLORIMETER

FILTER NUMBER Mμ	DILUTIONS OF DYE					CENTER SETTING
	1:6,000	1:12,000	1:15,000	1:18,000	1:30,000	
520	24.0	47.0	55.0	60.5	78.0	75.0
540	7.0	24.5	32.0	38.0	61.0	75.0

From Table II we see that a 4 u test (54.5) equals 1:15,000 dilution (55.0) on Table III at 520 mμ. A 4 u test contains six times 1:15,000 Gm. dye as 100 per cent toluidine blue, since a 4 u test contains 6 c.c. total solution. The decolorization due to 4 u of heparin equals 6 (1:6,000) − 6 (1:15,000) = 36:60,000 = 600:1,000,000 = 600 gamma. Therefore, 1 u heparin decolorizes 150 gamma of 100 per cent toluidine blue.

METHOD II

We have found that in method I there is a possible experimental error of ±50 gamma between triplicate tests. Assuming an error of 40 gamma in testing a solution diluted to 1 c.c. = 1 u, a 3 unit test would leave 590 or 510 gamma, and

a 4 unit test would leave 440 or 360 gamma of dye in solution. If a 3 unit test leaves 590 gamma and a 4 unit test leaves 440 gamma, the value of the solution tested would equal 0.92 u per cubic centimeter. If a 3 unit test leaves 510 gamma and a 4 unit test leaves 360 gamma, the value of the solution tested would equal 1.08 u per cubic centimeter. Referred back to a sample of 100 u per milligram, this would mean an error of ±8 u per milligram. In order to test weighed dry samples in concentrated solution, we developed the following procedure:

Procedure.—A measured portion of the sample is titrated in a beaker with a 1:2,000 solution of TB or AA made up by adding 0.8475 Gm. TB (NU-3) to 1,000 c.c. or 0.5747 Gm. AA (NAz-7) to 1,000 c.c. distilled water. To this solution add 0.05 c.c. increments, with swirling, from a 25 c.c. burette until precipitation starts, allow it to aggregate, and continue to add slowly, drop by drop.

TABLE IV

SAMPLE TITRATIONS WITH 0.05 PER CENT SOLUTIONS OF TOLUIDINE BLUE AND AZURE A

HEPARIN		TITRATIONS IN C.C.				COLORIMETRIC UNITS PER MG.
IDENTIFICATION	MG.	1	2	3	AVERAGE	
Na-50-110	0.454	13.1	13.15	13.1	13.1	96.2
Na-61-110	0.454	12.1	12.2	12.2	12.2	89.7
Na-60-110	0.454	12.0	11.8	11.9	11.9	87.3
Ba-61-100	0.500	13.1	13.1	13.3	13.2	88.0
Ba-62-100	0.500	11.5	11.6	11.5	11.5	76.7
Ba-43-4-100	0.500	12.2	12.25	12.15	12.2	81.3

3 ⅓ u = 1 c.c. of 1:2,000 TB; Na indicates sodium salt; Ba indicates barium salt.

TABLE V

COMPARISON BETWEEN ANTICOAGULANT AND COLORIMETRIC UNITS OF HEPARIN FROM VARIOUS SOURCES

HEPARIN IDENTIFICATION	ANTICOAGULANT UNITS PER MG.	COLORIMETRIC UNITS PER MG.
Lederle Laboratories		
Na-43-4	110	109
Na-45	110	102
Na-50	110	96
Na-61	110	90
Na-60	110	87
Ba-61	100	88
Ba-43-4	100	81
Ba-44	100	79
Ba-62	100	77
Ba63	100	76
Connaught Laboratories		
I	120	108
II	120	98
III	115	93
IV	110	108
V	100	76
Abbott Laboratories		
Na-4	145	106
Ba-1-PHi	95	90
Ba-3-PHi-II	90	90
Ba-2-PHi*	50	77
British Drug House†	85	95
	85	84
Løvens, Copenhagen† Na	85	84

*Dried at 80° C. and 40 mm. vacuum.
†Assayed and kindly supplied by Dr. W. C. Risser.

until a distinct blue solution equal to about 1:40,000 TB is observed among the heparin dye flocculation. The titration in cubic centimeters is multiplied by 3⅓ to obtain unitage in colorimetric units. Titration is materially assisted by bottom lighting under the beaker and it is extremely important that the precipitate be allowed to form slowly in order to avoid overtitration. The amount of sample plus titrating solution should not be greater than 75 c.c. at the end point, for, in the course of our work we find that certain concentrations are necessary to produce precipitation of the dye heparin complex. Considerable dilution before adding TB or the addition of TB of too great dilution will prevent precipitation. Various shades of blue mixed with violet will be the result no matter how great the excess of TB or AA over heparin. In titration at considerable dilution the deep violet color of the TB heparin or AA heparin complex masks the end point and makes a sharp end point impossible, except, possibly, a special filter in a photoelectric colorimeter might determine the end point accurately. However, since it is simpler to precipitate the interfering violet at the end point, we choose to do so. We find that a concentration of approximately 1:40,000 of TB or AA is necessary for complete precipitation.

Results.—From tests conducted on samples, we have found that a 100 unit sample of the purest heparin with regard to our reaction yielded a 30 c.c. titration with 1:2,000 TB. Therefore, we arbitrarily set 1 c.c. of 1:2,000 TB equal to 3⅓ u. One cubic centimeter of 1:2,000 TB equals 0.5 mg. TB; therefore, 1 u equals 0.15 mg. (or 150 gamma).

From triplicate titrations of heparin in Table IV it can be seen that a sharp end point is obtained and that the test is reproducible with rather high accuracy (±2 per cent), and over-all accuracy about ±5 per cent.*

In Table V the anticoagulant units and colorimetric units of heparin from various sources are compared.

DISCUSSION

Relationship Between Azure A and Toluidine Blue.—Our results indicate with regard to heparin that a solution of azure A of the same reactive strength as a solution of toluidine blue shows equal chromatic densities at various dilutions. We have found that a solution of azure A that will give, for example, a 30 c.c. titration with a certain sample of heparin when compared with a solution of toluidine blue that gives a 30 c.c. titration with the same sample of heparin will show the same chromatic concentration. Dr. Conn[2a] stated that toluidine blue has not been satisfactorily standardized, and he refers to the formula[11] published demonstrating that its composition is very similar to azure A. In view of the results we obtained, the question arises as to whether the difference between the two dyes is even as much as has been indicated by the published formulas. In this connection it was found that upon standing a shift in the absorption band occurs.[2a] From this Conn concluded that it would appear that toluidine blue can spontaneously change into azure A. However, such a particular chemical change seems to him to be improbable. He feels that there is another, yet undiscovered, explanation for this phenomenon. Old samples of toluidine blue in which this

*All weighings must be to the third significant figure to obtain ± 2 per cent accuracy.

change in color has occurred yielded the same results as azure A. These findings seem to indicate that it might be difficult to standardize any technique that calls for toluidine blue. However, the present toluidine blue (NU-3) of the National Aniline Chemical Company, which has been made in a way different from earlier lots, has to date not shown this change in color absorption. Our sample (NU-3), which was rechecked by Dr. Conn, did not show any change whatsoever in its absorption maximum and agrees entirely in its optical characteristics with the one he has on hand.*

The sample of NU-3 (dye strength 59 per cent) was compared to azure A (NAz-7) and the NAz-7 showed a dye strength of 87 per cent toluidine blue. It would appear that the NAz-7 has, for instance, 13 per cent impurities as compared to 41 per cent in the NU-3. We wonder if an appreciable amount of leuco products is present in the azure A as seems to be in toluidine blue according to information from the manufacturer.[7a] However, we do not know if leuco compounds have any effect upon the thiazins as to causing change in the chromophore group. Both azure A and toluidine blue gave reproducible results with heparin, and thus we feel that these dyes, as standardized by the Commission on Biological Stains, can be used interchangeably.

Comparison of Anticoagulant and Colorimetric Units of Heparin.—By our test the various samples contributed by different laboratories show variation up to 33 per cent of the label value. This suggests that either our test does not measure the anticoagulant value of the sample as a whole, or else the previous tests used were not based on as reproducible conditions as ours. It may be noted that various biological assay methods were used to determine the unit of heparin.†‡ However, the possibility is present that the heparin samples as prepared at the present time do not owe all their anticoagulant power to the heparin compound alone, but may be modified by plasma factors not identified[13] and possibly present in the albumin fraction.[14, 15] An alternative possibility is that there are present in the samples impurities which modify the toluidine blue or azure A reaction. Nevertheless, we feel, since other polysulfuric acid esters of polysaccharides with comparatively high molecular weight exhibit anticoagulant activity[16, 17] and also metachromatic change, as for instance in chondroitin sulfuric acid,[1, 18] they seem to be of similar chemical composition. Our data may not truly represent the physiological activity of the heparin samples, although the results are proportional to the anticoagulant activity as found by various workers. In this connection, a portion of a sample containing about 95 u per milligram was dried to constant weight, heating at 80° C., under low vacuum of 40 mm. and with a slow stream of dry air drawn over the powder. The anticoagulant activity of this portion was found to be 50 u. By comparing the colorimetric titration we found 90 u in the first portion, 77 u in the second. Although the discrepancy between both portions is about 3½ times larger with the biological assay method, the decrease of 13 units with our method is significant. In a test based on physiological standards, such as cat or ox blood, the

*Personal communication, January 16, 1942.

†Dr. Charles used a modification of Howell's method by Charles and Scott.[12] Dr. Meadows used a modification of the method of Reinert and Winterstein.[9] Dr. Risser used a modification of the method of Charles and Scott.[12]

‡Assays employed by Charles, Risser, and Meadows show accuracies as high as ± 5 per cent.

reliability of such a standard is always open to question. According to Jorpes[19] differences of 15 to 20 per cent were demonstrated with his method of standardization with ox blood. There are seasonal changes reported by him. For instance, he found the tendency to coagulation in the summer was at least twice as great as during the winter. Other factors are mentioned by Jorpes which may affect the coagulation time, such as temperature, species, and admixtures of thromboplastin (thrombokinase). Therefore, we feel justified, in view of the indefinite status of the unit of heparin, in establishing a unit on the basis of these reliably reproducible azure A-toluidine blue reactions with heparin.

Colorimetric Unit of Heparin.—We have demonstrated by Tables II and III that a 4 u test of a sample of the Connaught Laboratories containing 110 u per milligram, which exhibited the strongest colorimetric reaction, decolorized 150 gamma per Murray-Best unit. For convenience in computation we have chosen this sample to define the unit of heparin. Therefore, we present the colorimetric unit of heparin equaling the decolorization of 150 gamma of 100 per cent dye content of toluidine blue. It follows that this unit would equal 254 gamma of toluidine blue NU-3 or 172 gamma of azure A NAz-7. We discover in our titration method (method II) that, using 1 u equaling 150 gamma 100 per cent toluidine blue, our results correspond with results obtained by our decolorization method (method I).

SUMMARY

1. The toluidine blue reaction for heparin was developed by use of the Duboscq and Evelyn colorimeters and shown to be practical for assay purposes. The same reaction for heparin was found to be specific with azure A.

2. The relationship between azure A and toluidine blue is discussed. With heparin, azure A yielded 87 per cent dye content as toluidine blue compared to 59 per cent dye content toluidine blue.

3. A titration method of positive value for heparin assay was established which yielded rapid results, reproducible to ±2 per cent, over-all accuracy about ±5 per cent.

4. The comparison between anticoagulant and colorimetric units is discussed.

5. The colorimetric unit of heparin is defined. One unit of heparin decolorizes 150 gamma of 100 per cent toluidine blue dye content. This unit equals 254 gamma of toluidine blue NU-3 or 172 gamma of azure A NAz-7.

We wish to thank Dr. A. F. Charles of Toronto, Dr. C. M. Meadows of Pearl River, N. Y., and Dr. W. C. Risser of North Chicago for supplying us with their preparations of heparin. We also wish to thank Dr. H. J. Conn for his courteous cooperation.

REFERENCES

1. Copley, A. L.: The Excretion of Injected Heparin in the Urine of Mice and Dogs, Science 93: 478, 1941.
2. Copley, A. L., and Schnedorf, J. G.: The Rate of Excretion of Heparin in the Urine Following Its Intravenous Injection in the Anesthetized Dog, Am. J. Physiol. 133: 562, 1941.
2a. Personal communication, Oct. 29, 1941.
3. Jaques, L. B., and Charles, A. F.: The Assay of Heparin, Quart. J. Pharm. & Pharmacol. 14: 1, 1941.
4. Lison, L.: Etudes sur la métachromasie. Colorants métachromatiques et substances chromotropes, Arch. de biol. 46: 599, 1935.

5. Lison, L.: Une réaction micro et histochimique des esters sulfuriques complexes, la "réaction métachromatique," Bull. Soc. chim. biol. **18**: 225, 1936.
6. Jorpes, E., and Bergstroem, S.: Der Aminozucker des Heparins, Ztschr. physiol. Chem. **244**: 253, 1936.
7. Jorpes, E.: On Heparin, Its Chemical Nature and Properties, Acta med. Scandinav. **88**: 427, 1936.
8. Charles, A. F., and Todd, A. R.: Observations on the Structure of the Barium Salt of Heparin, Biochem. J. **34**: 112, 1940.
9. Reinert, M., and Winterstein, A.: Contribution to the Study of Heparin, Arch. internat. de pharmacodyn. et de thérap. **62**: 47, 1939.
10. Conn, H. J.: Biological Stains. A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory, ed. 3, Geneva, N. Y., 1936, Commission on Standardization of Biological Stains, page 76.
11. Murray, D. W. G., and Best, C. H.: Heparin and Thrombosis, J. A. M. A. **110**: 118, 1938.
12. Charles, A. F., and Scott, D. A.: Studies on Heparin. IV. Observations on the Chemistry of Heparin, Biochem. J. **30**: 1927, 1936.
13. Copley, A. L.: On the Thromboplastic Activity of Brain and Skin Extracts, Am. J. Physiol. **137**: 178, 1942.
14. Jaques, L. B., and Mustard, R. A.: Some Factors Influencing the Anticoagulant Action of Heparin, Biochem. J. **34**: 153, 1940.
15. Ziff, M., and Chargaff, E.: Studies on the Chemistry of Blood Coagulation. XI. The Mode of Action of Heparin, J. Biol. Chem. **136**: 689, 1940.
16. Bergstroem, S.: Ueber Polysaccharidesterschwefelsäuren mit Heparinwirkung, Ztschr. physiol. Chem. **238**: 163, 1936.
17. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M.: Studies on the Chemistry of Blood Coagulation. II. On the Inhibition of Blood Clotting by Substances of High Molecular Weight, J. Biol. Chem. **115**: 155, 1936.
18. Jorpes, E.: Ueber das Heparin und seine Anwendung als Ersatzmittel für Oxalat und Zitrat in der Klinik, Acta med. Scandinav., suppl. 89, page 139, 1938.
19. Jorpes, J. E.: Heparin. Its Chemistry, Physiology and Application in Medicine, London, 1939, Humphrey Milford, Oxford University Press, page 46.

THE QUANTITATIVE ESTIMATION OF GLUCURONATES IN URINE*

WM. DEICHMANN, PH.D., CINCINNATI, OHIO

THE need has long been felt for a reliable quantitative method of estimating glucuronates in urine. Such a method would be of special value in determining the extent of exposure of workmen to any compound which had been found to be detoxified by conjugation with glucuronic acid.

Some of the methods reported in the literature require from eighteen to twenty-four hours for a single analysis (Sauer,[1] Kakinuma[2]); others, based on the formation and distillation of furfural, are disturbed if glucides are present, and are not sufficiently precise in dealing with amounts less than 2 mg. (Tanabe,[3] Fürth and Peschke[4]). Venning[5] employed a gravimetric procedure but obtained only approximately correct results. Florkin,[6] and Maughan, Evelyn, and Browne[7] have developed a quantitative method from Tollens'[8] qualitative naphthoresorcinol test, but single estimations were found unreliable. Recently, Meyer, Block, and Chaffee[9] have reported a method for the estimation of uronic acids in pure solutions.

*From the Kettering Laboratory of Applied Physiology in the College of Medicine of the University of Cincinnati.

Received for publication, July 11, 1942.

Principle of the Method.—The method employed here is also a modification of Tollens' original test, but, in contrast to similar procedures previously reported, it makes use of a preliminary hydrolysis at 75° C., a great excess of naphthoresorcinol, and a hydrochloric acid-naphthoresorcinol mixture heated to 50° C. Further, the final estimation is carried out with a photoelectric spectrometer or with a photoelectric colorimeter.

Experiments have shown that few compounds will interfere in this determination and that an occasional urine sample requires preliminary treatment. If urine contains no pentose or glucose, then the regular procedure is followed. However, if the concentration of pentose is found to be about 0.03 per cent or that of glucose is about 0.3 per cent, the sample analyzed should then be reduced to about 0.1 ml., and when the concentration of these sugars exceeds these limits, the urine samples should then be freed of them prior to hydrolysis. The presence of 1.0 per cent protein or 0.2 per cent ascorbic acid does not interfere. (The normal excretion of ascorbic acid ranges from 30 to 50 mg. in twenty-four hours,[11] which is not in excess of 0.025 mg. in 0.5 ml. of urine. It was found that when 0.5 mg. of ascorbic acid and 0.12 mg. of glucuronic acid were added to a 0.5 ml. sample of human urine, the recoveries of glucuronic acid ranged from 0.124 to 0.132 mg.)

Reagents Required.—Hydrochloric acid (19 Gm. in 100 ml.).

Naphthoresorcinol* (10 Gm. in 100 ml. of 95 per cent ethyl alcohol). This reagent is centrifuged or filtered and will remain stable for five days if kept in a cool place protected from light.

Ether, C.P.

METHOD OF ANALYSIS

(1) *Determining the Need for Removing Interfering Substances.*—The presence of glucose is readily determined by Benedict's qualitative test. Amounts of ascorbic acid sufficient to cause interference may be suspected only in cases of vitamin medication. Testing for pentoses may be carried out by Tollens' test slightly modified as follows: To 1 ml. of urine and 1 ml. of concentrated hydrochloric acid is added 0.1 ml. of 10 per cent naphthoresorcinol in 95 per cent ethyl alcohol. The mixture is boiled for one minute in a test tube with constant shaking, permitted to stand at room temperature for four minutes, cooled under the tap, and extracted with 10 ml. of ether. When the urine is free of pentose, the ether is purple (with the exception of unusual cases that contain no glucuronic acid at all); in the presence of 0.1 per cent pentose the ether is colorless, while intermediate concentrations give various shades of purple. (This unusual behavior seems to result from the speed of reaction of pentose with naphthoresorcinol. The latter appears to be faster than the speed with which glucuronic acid and naphthoresorcinol react. Hence if pentose and glucuronic acid are both present in a sample of urine, pentose will react first, forming a brownish-black ether-insoluble pigment; the absence of color in the ether layer is then due to the fact that the naphthoresorcinol has been used up. The preliminary hydrolysis in the quantitative procedure makes a large amount of "free" glucuronic acid available for immediate reaction. The high concentra-

*Purchased from B. L. Lemke, 248 West Broadway, New York City.

tion of naphthoresorcinol is needed, because this compound reacts not only with glucuronic acid but probably with many impurities; an excess furnishes enough for reaction with the urinary constituents that form ether-insoluble substances and for the reaction with glucuronic acid whereby the purple ether-soluble color is produced. These two factors are mainly responsible for the fact that glucuronic acid can be satisfactorily determined in relatively large urine samples [0.5 ml.] without removal of interfering substances. To study the color, it is best to set up a few specimens of normal urine to which have been added known amounts of arabinose.)

(2) *Removal of Interfering Substances.*—The removal of interfering substances is accomplished by precipitation with lead acetate in slightly acid solution, as suggested originally by Quick,[10] and the precipitation of conjugated glucuronates with basic lead acetate, according to Tollens.[5]

A small quantity (0.2 to 0.4 ml.) of the urine to be tested is measured into a 15 ml. conical centrifuge tube, diluted to 2 ml. with water, and made acid to litmus with 1 to 3 drops of glacial acetic acid. Then, 0.2 ml. of 5 per cent lead acetate is added and the sample is mixed and centrifuged. Tests should be made for complete precipitation of interfering substances by the addition of a drop of lead acetate. If precipitation is found to be incomplete, an additional 0.1 ml. of lead acetate solution is added, the mixture is shaken and again centrifuged. This procedure is repeated until precipitation is complete. The supernatant fluid is poured into a similar centrifuge tube and 0.3 ml. of 10 per cent sodium hydroxide is added. If a precipitate does not form, more sodium hydroxide is added until a permanent precipitate is obtained. Then 3 ml. of the basic lead acetate solution* are added, and the contents are mixed and centrifuged. Tests should be made for complete precipitation of glucuronates by the further addition of a drop of basic lead acetate. When precipitation is complete, the supernatant fluid is discarded and the precipitate is washed by thoroughly stirring it with 10 ml. of water. The mixture is centrifuged again, the supernatant liquid is discarded. The residue is transferred quantitatively, employing 5 ml. of concentrated hydrochloric acid followed by 5 ml. of water, into a 50 ml. graduated cylinder and then incubated for fifteen minutes at 75° C. One milliliter of 10 per cent naphthoresorcinol is added, and the cylinder is allowed to stand for ninety minutes in a water bath maintained at 50° C. After cooling under the tap, the solution is extracted with ether and the remainder of the determination is carried out as described below.

(3) *Analysis.*—(The analysis is started at this point if interfering amounts of glucose, pentose, protein, or ascorbic acid are known to be absent.)

The sample of urine is mixed well, and its total volume is determined. In the case of human or dog urine, 0.3 to 0.5 ml., and of rabbit's urine, 0.2 to 0.4 ml., is then pipetted into a 50 ml. glass-stoppered graduated cylinder and diluted with water to 5 ml. After the addition of 0.2 ml. of 10 per cent hydrochloric acid, the whole is kept for forty-five minutes in a water bath at 75° C.; 5 ml. of concentrated hydrochloric acid and 1 ml. of 10 per cent naphthoresorcinol are then added, and the cylinder is allowed to stand for ninety minutes in a water

*Prepared by heating 10 Gm. of dibasic lead acetate with 200 ml. of water; after boiling and stirring for a few minutes, the solution is allowed to cool and is filtered.

bath maintained at 50° C. After cooling under the tap, the solution is extracted with 10 ml. of ether by shaking the cylinder vigorously for five seconds. After separation into two layers, sufficient ether is added to bring the ether volume to exactly 15 ml., and it is mixed by gently rotating the cylinder. Addition of the second portion of ether will completely clear up any slight turbidity caused by water in the upper layer.

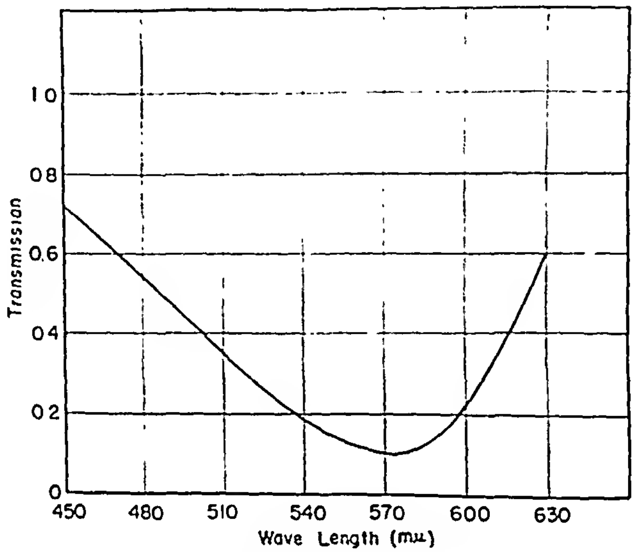


Fig. 1.—Transmission curve of the ether extract.

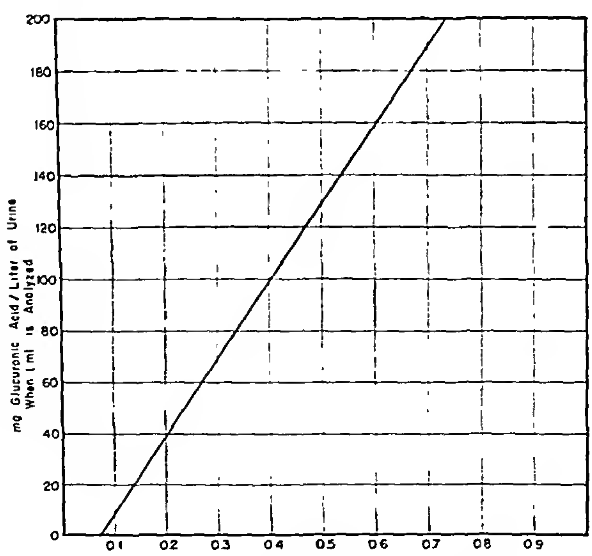


Fig. 2.—Glucuronic acid. Concentration versus density measured with the spectrophotometer at 570 mμ employing 10 mm. matched cells.

(4) *Colorimetric Reading.*—The colored ether is now pipetted into a glass-stoppered flask and is ready for spectrophotometric analysis, which is carried out by using 10 mm. matched cells, with the instrument set at the wave length

of 570 mμ. The final estimation may also be carried out, with equally good results, with a Leitz photoelectric colorimeter employing the green filter No. F-401.

Fig. 1 gives the transmission curve of the pigment formed under the conditions outlined in this procedure.

Fig. 2 gives the relationship between density and concentration of glucuronic acid per liter of urine when the photoelectric spectrometer is employed.

Fig. 3 gives the relationship between concentration of glucuronic acid per liter of urine and readings with the photoelectric colorimeter.

Figs. 2 and 3 were prepared by plotting the readings obtained when known aqueous solutions of pure glucuronic acid (or of a conjugated glucuronate) were analyzed by this procedure.[12]

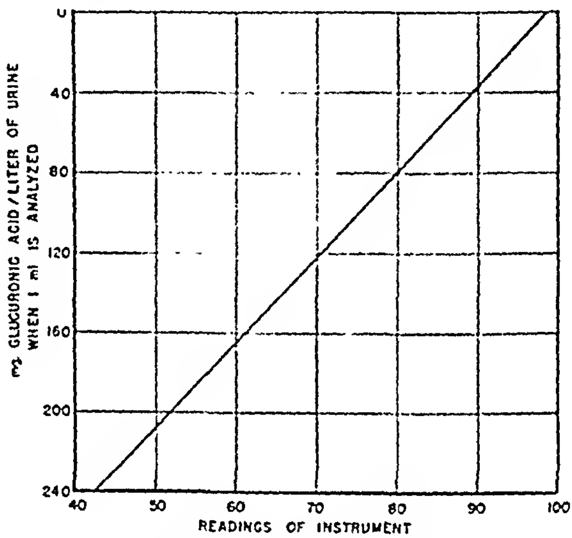


Fig. 3.—Glucuronic acid. Concentration versus reading with the Leitz photoelectric colorimeter with green filter No. F-401.

(5) *Calculation.*—The following example will serve to illustrate the calculation: if 0.25 ml. from a total volume of 280 ml. is analyzed, and a concentration of 70 obtained from Fig. 2 or Fig. 3, then:

$$\frac{70}{1{,}000} \text{ mg.} \times \frac{1}{0.25} \times 280 \rightarrow 78.4 \text{ mg. glucuronic acid/total volume.}$$

(6) *Certain Practical Considerations.*—

(a) The alcoholic naphthoresorcinol solution employed must be *perfectly clear.* The amounts of impurities in this compound vary; it is, therefore, advisable to plot new curves with each new lot of naphthoresorcinol.

(b) The colored ether solution is stable for about one hour; it should be protected from direct sunlight. (Prolonged standing causes an increase in the intensity of the color.)

(c) The analysis must be carried out without delay after the addition of hydrochloric acid. If it should be found necessary to remove interfering substances from the urine, a convenient point to interrupt the analysis comes after the precipitation of glucuronates with basic lead acetate.

(d) An excess of acid lead acetate must be avoided because this will result in loss of glucuronates.

(e) Normal human urine suffers no loss of glucuronic acid when permitted to stand for a week at room temperature without preservatives.

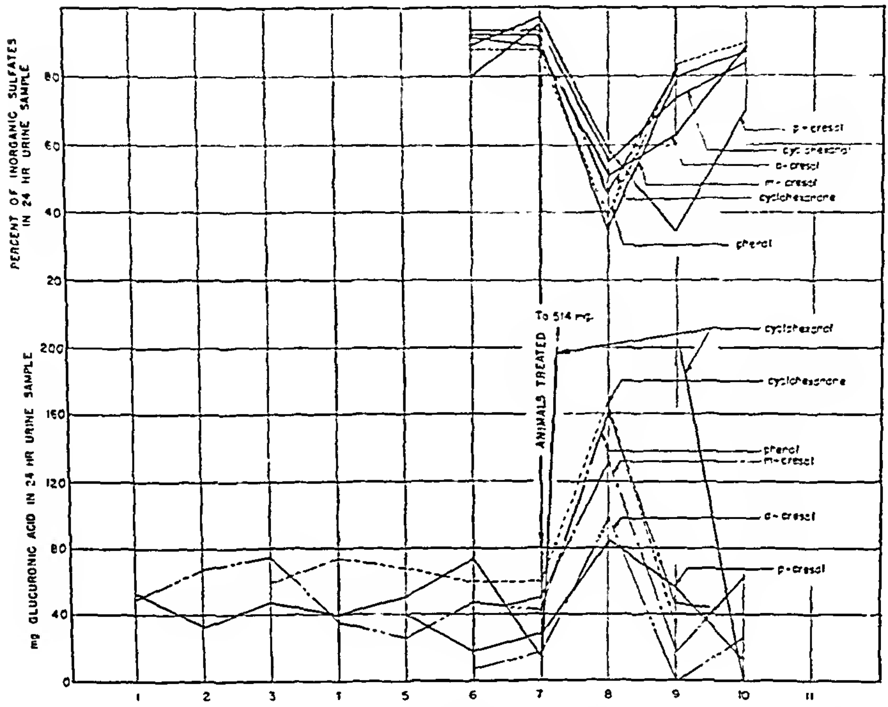


Fig. 4.—Excretion of inorganic sulfates and glucuronic acid in the urine of rabbits following a single oral administration of about half the lethal dose of phenol, o-, m-, and p-cresol, cyclohexanol, and cyclohexanone, respectively.

EXPERIMENTAL

Table I summarizes the recoveries of free and conjugated glucuronic acid added to normal human or rabbit urine and analyzed without previous removal of interfering substances. The recoveries are satisfactory. Table II gives the amounts of free and conjugated glucuronic acid recovered after their addition to urine containing glucose or arabinose; here again the urine was not subjected to preliminary treatment. It can be seen that the presence of 0.03 per cent arabinose or 0.5 per cent glucose does not interfere with recovery of the conjugated compound; the recovery of free glucuronic acid is slightly less complete. Table III shows that following the removal of interfering substances from the urine, as outlined above, 0.25 per cent arabinose added to the urine is removed to a sufficient degree to enable recovery of the conjugated glucuronates; a slight loss of glucuronates is noted, however, when urine contains 0.5 per cent of this pentose. Table IV summarizes the excretion of glucuronic acid in twenty-four-hour specimens from normal men who were under no dietary restrictions. The daily excretions ranged from 65 to 239 mg. These amounts bore no relationship to the total volume of urine excreted. A young dog on a diet consisting

TABLE I

RECOVERY OF FREE AND CONJUGATED GLUCURONIC ACIDS ADDED TO NORMAL URINE*

SAMPLE	COMPOUND ADDED (MG.)	COMPOUND RECOVERED (MG.)
	Glucuronic acid	
0.5 ml. human urine	0.06	0.07
0.5 ml. human urine	0.12	0.13
0.5 ml. human urine	0.18	0.19
0.25 ml. rabbit urine	0.12	0.10
0.25 ml. rabbit urine	0.18	0.17
0.4 ml. rabbit urine	0.12	0.15
	Borneolglucuronic acid	
0.5 ml. human urine	0.06	0.07
0.5 ml. human urine	0.06	0.05
0.5 ml. human urine	0.12	0.14
0.5 ml. human urine	0.18	0.19
0.5 ml. human urine	0.18	0.17
0.1 ml. rabbit urine	0.12	0.12
0.1 ml. rabbit urine	0.24	0.21
	Mentholglucuronic acid	
0.25 ml. human urine	0.06	0.07
0.25 ml. human urine	0.12	0.14
0.25 ml. human urine	0.24	0.24
0.25 ml. human urine	0.24	0.20
0.4 ml. rabbit urine	0.13	0.10
0.1 ml. rabbit urine	0.06	0.08
0.1 ml. rabbit urine	0.18	0.18
0.1 ml. rabbit urine	0.24	0.21

*Interfering substances were not removed.

TABLE II

RECOVERY OF GLUCURONIC ACID ADDED TO URINE CONTAINING GLUCOSE OR ARABINOSE*

SAMPLE 0.1 ML. OF RABBIT URINE CONTAINING	COMPOUND ADDED (MG.)	COMPOUND RECOVERED (MG.)
	GLUCURONIC ACID	
0.3% glucose	0.06	0.05
0.3% glucose	0.12	0.11
0.3% glucose	0.18	0.19
0.5% glucose	0.03	0.02
0.5% glucose	0.06	0.05
0.5% glucose	0.12	0.09
0.03% arabinose	0.03	0.02
0.03% arabinose	0.06	0.04
0.03% arabinose	0.12	0.10
0.1% arabinose	0.18	0.14
0.1% arabinose	0.13	0.09
0.5% arabinose	0.12	0
0.5% arabinose	0.12	0.04
0.5% arabinose	0.12	0.03
	BORNEOLGLUCURONIC ACID	
0.5% glucose	0.06	0.06
0.5% glucose	0.12	0.14
0.5% glucose	0.18	0.19
0.5% glucose	0.18	0.17
0.5% glucose	0.18	0.16
0.5% glucose	0.18	0.19
0.5% glucose	0.18	0.17
0.03% arabinose	0.12	0.11
0.03% arabinose	0.18	0.17
0.05% arabinose	0.12	0.09
0.05% arabinose	0.18	0.15
0.1% arabinose	0.12	0.08
0.1% arabinose	0.18	0.13
0.5% arabinose	0.18	0

*Interfering substances were not removed.

TABLE III

RECOVERY OF GLUCURONIC ACID ADDED TO URINE AFTER REMOVAL OF ARABINOSE AND GLUCOSE

SAMPLE 0.1 ML. OF RABBIT URINE CONTAINING	COMPOUND ADDED (MG.)	COMPOUND RECOVERED (MG.)
	GLUCURONIC ACID	
0.1% arabinose	0.13	0.11
0.25% arabinose	0.12	0.08
0.25% arabinose	0.12	0.09
0.5% arabinose	0.12	0.09
0.5% arabinose	0.12	0.07
0.5% glucose	0.12	0.11
	BORNEOLGLUCURONIC ACID	
0.25% arabinose	0.06	0.05
0.25% arabinose	0.06	0.04
0.25% arabinose	0.12	0.13
0.25% arabinose	0.12	0.13
0.25% arabinose	0.12	0.10
0.25% arabinose	0.12	0.10
0.5% arabinose	0.06	0.06
0.5% arabinose	0.06	0.04
0.5% arabinose	0.12	0.08
0.5% arabinose	0.12	0.08
0.5% arabinose	0.12	0.10

TABLE IV

GLUCURONIC ACID CONTENT OF NORMAL HUMAN URINE*

(The volume of samples analyzed ranged from 0.25 to 0.5 ml.)

CASE	VOLUME OF THE 24-HOUR SPECIMEN (ML.)	GLUCURONIC ACID IN THE 24-HOUR URINE SPECIMEN (MG.)
LD	370	117
LD	465	114
FW	500	125
RH	540	139
HP	600	65
WD	660	128
HD	750	83
HP	760	84
RH	775	101
WD	795	139
WD	795	186
LD	800	105
RH	845	130
WH	870	158
WD	885	224
FW	925	159
HD	945	120
WH	950	163
WD	950	104
WD	1,003	123
HP	1,070	75
AB	1,100	239
HD	1,100	154
FF	1,260	86
GN	1,350	142
GN	1,565	139
WD	1,640	164
GN	1,945	154
GN	2,025	146

*The urine was not subjected to preliminary treatment because specimens did not contain glucose, pentose, or protein in interfering quantities and the subjects had not received ascorbic acid as medication.

of beef heart and "kibbles" (dried dog food) excreted daily for six days from 24 to 108 mg. of glucuronic acid, and rabbits on a diet of Purina pellets excreted daily for eighteen days from 15 to 75 mg.

Fig. 4 shows the drop in inorganic sulfates and the corresponding increased output of glucuronic acid in rabbits following administration of half the lethal oral dose of phenol, o-, m-, and p-cresol, cyclohexanone, and cyclohexanol, respectively.

SUMMARY

1. A quantitative procedure is presented for the estimation of glucuronates in urine. The method is sufficiently reliable for clinical and industrial use.

2. The daily output of glucuronates for men on an unrestricted diet ranged from 65 to 239 mg. per twenty-four hours. A dog excreted from 24 to 108 mg. and rabbits from 15 to 75 mg. for one-day periods.

3. The administration of about half the lethal oral dose of phenol, o-, m-, and p-cresol, cyclohexanol, and cyclohexanone, respectively, produces, in addition to the well-known drop in the output of inorganic sulfates, a sharp rise in the excretion of glucuronates.

The borneolglucuronic and the mentholglucuronic acids were prepared and kindly supplied by Dr. Armand T. Quick of Marquette University School of Medicine; the glucuronic acid was kindly supplied for these studies by J. R. Harrower of the University of Wisconsin.

REFERENCES

1. Sauer, J.: Die quantitative Glykuronsäurebestimmung im Harn ist eine Leberfunktionsprüfung, Klin. Wchnschr. 9: 2350, 1930.
2. Kakinuma, Saburo: Microquantitative Estimation of Glucose, Free and Conjugated Glucuronic Acid. A Method of Determination of Glucuronic Acid in Presence of Glucose in Human and Rabbit Urine, J. Pharm. Soc. Japan 59: 635, 1939; 60: 424, 1940.
3. Tanabe, Yasuhisa: Biochemical Studies on Carbohydrates. XXX. Our Iodometric Method for the Determination of Glucuronic Acid, J. Biochem. (Japan) 27: 251, 1938.
4. Fürth, Otto, and Peschke, Karl: Über Mikrobestimmung der Glucuronsäure, Biochem. Ztschr. 287: 365, 1936.
5. Venning, Eleanor H.: Further Studies on the Estimation of Small Amounts of Sodium Pregnanediol Glucuronidate in Urine, J. Biol. Chem. 126: 595, 1938.
6. Florkin, Marcel: Principes d'une méthode de dosage de l'acide glucuronique, Compt. rend. Soc. de biol. 126: 916, 1937.
7. Maughan, G. B., Evelyn, K. A., and Browne, J. S. L.: A Method for the Quantitative Estimation of Glucuronic Acid and Conjugated Glucuronides, J. Biol. Chem. 126: 567, 1938.
8. Tollens: Handbuch der Kohlenhydrate, ed. 3, Leipzig, 1914.
9. Meyer, Karl, Block, Henry S., and Chaffee, Eleanor: The Colorimetric Determination of Uronic Acids, Federation Proc. 1: 125, 1942.
10. Quick, A. J.: The Preparation and Study of β, d-Glycuronic Acid Monobenzoate (Benzoyl Glycuronic Acid), J. Biol. Chem. 69: 547, 1926.
11. Best, C. H., and Taylor, N. B.: The Physiological Basis of Medical Practice, Baltimore, 1940, Williams & Wilkins Company.
12. Quick, A. J.: A Method for the Quantitative Determination of Menthol Glycuronic Acid in Urine, J. Biol. Chem. 61: 667, 1924.

THE DETERMINATION OF BROMIDES AND CHLORIDES IN BIOLOGICAL MATERIALS AND AN ACCURATE CLINICAL METHOD FOR DETERMINING BROMIDE IN SMALL AMOUNTS OF BLOOD*

LEON A. GREENBERG, NEW HAVEN, CONN.

IN CARRYING out a series of investigations on the effects of ingested bromides, the needs arose for: (1) a rapid method for determining accurately both bromides and chlorides in a single wet sample of tissue or blood; and (2) a rapid and reliable clinical method for determining the concentration of bromide in 0.1 c.c. of blood as drawn from a needle prick. Trial of the various methods for determining bromides and chlorides reported in the literature showed that none fully satisfied these needs. Two procedures which did satisfy them were, therefore, developed and are reported here. The findings from analyses made on predetermined amounts of bromide and chloride are given to show the accuracy of the methods and in addition the results from a series of determinations on the comparative bromide content of whole blood and serum.

All determinations of bromide in biological material are carried out in two stages: (1) the treatment of the material to obtain the bromide in a form and medium suitable for estimation; and (2) the estimation of the bromide. Three general methods are available for carrying out the first stage: (1) deproteinization; (2) destruction of the dried organic material by alkaline ashing; and (3) destruction by acid digestion.

TREATMENT OF MATERIAL

Deproteinization can be applied only to fluids; it imposes limitations also on the methods for estimating the bromide. It has been used for the electrometric titration and colorimetric estimation of bromide in blood, serum, and cerebrospinal fluid, but not previously for direct chemical estimation, as in the clinical method to be described later.

Alkaline ashing is the treatment most commonly used. A review of the literature shows, however, that it has given unsatisfactory results in the hands of many investigators. The method in general requires the use of dried material and is open to errors from spattering, overheating, and transfer of materials. Neufield[1] has described a modification of the Francis and Harvey method with which he obtained accurate recoveries, but the procedure required six to eight hours for a single determination. Brodie and Friedman[2] have used a type of ashing described by Kendall[3] and report that a complete determination may be made in about two hours with accurate recoveries. In this laboratory we have not been able to obtain consistently accurate results with alkaline ashing procedures.

*From the Laboratory of Applied Physiology, Yale University, New Haven, Conn.
Received for publication, July 15, 1942.

Acid digestion, except in the form of the closed Carius[4] procedure, has been used much less commonly than the alkaline ashing. It has the advantage that it is a wet digestion and it is rapid. It has, however, the particular disadvantage that the bromide must first be converted to an insoluble salt to prevent loss and then after the digestion reconverted to soluble bromide for estimation. Hartner,[5] failing to obtain satisfactory results with alkaline ashing, used concentrated nitric acid in the presence of silver nitrate. He converted the silver bromide to soluble bromide by treatment with zinc and sodium sulfide. Doering,[6] using the closed Carius method, found that this conversion was not always quantitative and employed zinc only. The procedure took many hours and any advantage of speed of the acid digestion over the alkaline ashing was lost. The procedure to be described in the next section as a laboratory method for the determination of bromide and chloride in tissue employs acid digestion but successfully overcomes the slow conversion of the silver halides with zinc by dissolving them in sodium thiosulfate solution and recovering the soluble bromide.

ESTIMATION OF BROMIDE

Four methods, each with various advantages and disadvantages, are commonly used for estimating bromides after treatment of the material: (1) colorimetric determination; (2) electrometric titration; (3) the liberation and estimation of bromide; and (4) the oxidation of the bromide to bromate and determination by iodometric titration.

For colorimetric determination of bromide the reagents used have been fluorescein,[7] fuchsin,[8] and gold chloride.[9] All colorimetric methods have proved poor because of their lack of sensitivity, inaccuracy, and nonspecificity. A method using gold chloride first described by Walter[9] and later modified by Wuth[10] and by Diethelm[11] has, in spite of its inaccuracy and unreliability, been used in many clinical investigations because of its simplicity.

Electrometric titration of bromide requires either that the bromide be present in concentrations approximating that of chloride present or the preliminary removal of most of the chloride. Its usefulness is correspondingly limited.

Several methods have been described for the oxidation of bromide to free bromine.[12-14] All of them necessitate an exact regulation of conditions to avoid the simultaneous formation of chlorine, incomplete oxidation of bromides, or loss of bromine.[1] All the procedures are time-consuming.

The oxidation of bromide to bromate is quantitative in the presence of chloride and the procedure for carrying it out is rapid and simple. The method originating with van der Meulen,[15] and modified by Kolthoff and Yutzy,[16] involves the oxidation of bromide to bromate by the use of hypochlorite according to the reaction:

$$3\ ClO^- + Br^- = BrO_3^- + 3\ Cl^-.$$

After the oxidation is complete, the excess hypochlorite is reduced with sodium formate. The addition of iodide to the bromate in acid solution results in the liberation of six equivalents of iodine, thus giving a sixfold increase in sensitivity over methods in which the bromine is titrated directly. This oxidation procedure has been used successfully by Dixon[17] and by Brodie and Friedman.[2]

LABORATORY METHOD FOR DETERMINING BROMIDE AND CHLORIDE IN BIOLOGICAL MATERIALS

The method described here has proved to be rapid and highly accurate; it avoids many difficulties inherent in methods previously described and permits the determination of both bromide and chloride on a single sample of material. The material is digested with nitric acid in the presence of a known quantity of silver nitrate. The digestion requires about fifteen minutes. The fluid containing the excess of silver nitrate is separated from the precipitate of silver halides and is titrated according to the procedure of Volhardt; from the value obtained the total halogen content of the material is calculated. The precipitate of silver halides is then dissolved in a solution of sodium thiosulfate; the thiosulfate is destroyed and its products are removed by heating with hydrochloric acid in the presence of metallic zinc. Metallic silver is precipitated. The dissolved zinc is precipitated with sodium carbonate and the filtrate contains all the halides in the form of sodium salts. The bromide is then oxidized by the method of Kolthoff and Yutzy[16] and the bromate is determined iodometrically. The detailed steps of the analytical procedure are as follows:

To the weighed sample (about 1 Gm.) of biological material in a 125 c.c. Erlenmeyer flask there are added exactly 3 c.c. of 0.05 N silver nitrate solution in concentrated nitric acid and also 25 c.c. of concentrated nitric acid. The mixture is placed on a hot plate for fifteen minutes for digestion. Saturated potassium permanganate solution is then added, drop by drop, until its color persists for several minutes; a few drops of 20 per cent sodium oxalate solution are added to remove the excess permanganate. The flask is then cooled under water flowing from a tap and placed in a rack so tilted that the precipitate of silver halides settles in a small area. The clear supernatant fluid is decanted into a second Erlenmeyer flask; the precipitate is washed twice with 10 c.c. of water from a washing bottle and the decanted fluid is added to that in the second flask. The silver nitrate in this fluid is titrated with thiocyanate and ferric alum after the method of Volhard.[18] The silver nitrate, as determined, when subtracted from that added, gives the total halide content of the material under analysis.

The bromide in the precipitate is then determined as follows: The precipitate is dissolved in 2 c.c. of 20 per cent sodium thiosulfate solution. Twenty cubic centimeters of distilled water are added and 10 c.c. of N hydrochloric acid and the mixture is boiled for five minutes. The flask is then removed from the hot plate and a pinch of powdered zinc is added; the mixture is then boiled vigorously for three minutes to remove the hydrogen sulfide formed. The flask is then removed from the heat and 5 c.c. of 20 per cent potassium carbonate are added to the solution which is then cooled and washed into a volumetric container to 50 c.c. volume. The solution is then filtered and 40 c.c. of the filtrate are placed in a 125 c.c. Erlenmeyer flask.

By the above procedure the silver bromide is converted to a soluble bromide which is then estimated by conversion to bromate followed by iodometric titration after the general procedure of Kolthoff and Yutzy as follows: 2 N hydrochloric acid is added until the acid is in slight excess. Approximately 2.5 Gm. of sodium dihydrogen phosphate and 6 c.c. of sodium hypochlorite solution (1 N in 0.1 N sodium hydroxide) are added, and the mixture is boiled gently for five min-

utes. Five cubic centimeters of 50 per cent sodium formate solution are added. The mixture is then boiled vigorously for five minutes and cooled, after which 5 Gm. of sodium dihydrogen phosphate, 20 c.c. of 2 N hydrochloric acid, 3 drops of 10 per cent ammonium molybdate solution and 1 Gm. of potassium iodide are added. The liberated iodine is titrated with 0.005 N thiosulfate; the titration is completed in the presence of 5 c.c. of 1 per cent starch solution. A blank determination is run whenever new reagents are made up, and the volume of thiosulfate used in this titration is subtracted from that used in analysis. Each cubic centimeter of thiosulfate is equivalent to 66.6 micrograms of bromine in the material used for analysis.

TABLE I

RECOVERIES OF BROMIDE AND CHLORIDE FROM AQUEOUS SOLUTIONS

BROMIDE ADDED (MG./GM.)	CHLORIDE ADDED (MG./GM.)	BROMIDE FOUND (MG./GM.)	CHLORIDE FOUND (MG./GM.)
0.388	1.52	0.383	1.55
		0.389	1.48
		0.387	1.53
	3.01	0.379	3.02
		0.382	3.01
		0.385	3.00
0.776	1.52	0.770	1.54
		0.778	1.52
		0.775	1.54
	3.01	0.771	3.03
		0.779	3.06
		0.780	3.02
1.552	1.52	1.548	1.51
		1.517	1.51
		1.551	1.55
	3.01	1.555	3.00
		1.554	3.01
		1.552	3.04
2.330	1.52	2.330	1.56
		2.321	1.52
		2.335	1.50
	3.01	2.321	3.03
		2.332	3.02
		2.336	3.05

In Table I are given the results from a series of 24 determinations made on four different aqueous solutions containing known amounts of bromide and chloride. The range of errors for bromide is +0.006 to −0.009 mg. and the algebraic mean is −0.00015; the corresponding values for chloride are +0.04, −0.04, and −0.004.

In Table II are given the results from a series of 12 determinations made on single specimens of blood to which known amounts of chloride and bromide were added. The amount of bromide and chloride in the blood before addition was taken as the average for six determinations. The range of error for bromide is +0.004 to −0.008, and the algebraic mean is −0.0015.

In Table III are given the results of triplicate analyses made on various tissues of an animal previously given sodium bromide. The range of errors in any one series of determinations is 0.005 mg. for bromide and 0.03 mg. for chloride.

TABLE II

RECOVERIES OF BROMIDE AND CHLORIDE FROM BLOOD

(In excess of normal content*)

BROMIDE ADDED (MG./GM.)	CHLORIDE ADDED (MG./GM.)	BROMIDE FOUND (MG./GM.)	CHLORIDE FOUND (MG./GM.)
0.388	1.52	0.389	1.51
0.388	1.52	0.380	1.52
0.388	1.52	0.385	1.55
0.388	1.52	0.388	1.50
0.388	1.52	0.387	1.55
0.388	1.52	0.381	1.54
1.552	1.52	1.548	1.53
1.552	1.52	1.551	1.50
1.552	1.52	1.550	1.52
1.552	1.52	1.555	1.52
1.552	1.52	1.552	1.54
1.552	1.52	1.556	1.50

*Average of six determinations: bromide 0.005 mg. per gram and chloride 5.74 mg. per gram.

TABLE III

RECOVERIES OF BROMIDE FROM TISSUES

TISSUE	BROMIDE PER GRAM WET TISSUE (MG.)	CHLORIDE PER GRAM WET TISSUE (MG.)
Blood	1.742	2.93
	1.747	2.91
	1.743	2.90
Liver	0.722	1.23
	0.719	1.20
	0.720	1.22
Spleen	0.891	1.61
	0.888	1.62
	0.889	1.58
Muscle	0.520	0.88
	0.520	0.90
	0.522	0.88
Heart	0.643	1.11
	0.646	1.09
	0.646	1.10

CLINICAL METHOD FOR DETERMINING BROMIDES

Many procedures described in the literature for the determination of bromide in blood are called micromethods. "Micro" as used in these instances refers to the minute amounts of bromide estimated and not the amount of blood used for the analysis. To the clinician, however, a micromethod is usually one by which clinically important concentrations of the substance being estimated can be determined on a "micro" sample. The methods previously reported for estimating the bromide in blood require 3 to 10 c.c. and hence necessitate venepuncture, and even with these large samples the clinical methods ordinarily employed are so inaccurate as to render them little more than indicators of the general magnitude of the bromide present. The method described here requires only 0.1 c.c. of blood which can readily be obtained by puncture of the

skin of the finger tips or ear lobes. The general procedures consist in deproteinization of the blood, the oxidation of the bromide in the filtrate to bromate, and the iodometric titration of the bromate. The procedure in detail is as follows:

The 0.1 c.c. of blood is discharged directly from the pipette in which it is drawn into 6.5 c.c. of 0.45 per cent zinc sulfate solution in a test tube. One-half cubic centimeter of 0.1 N sodium hydroxide solution is then added and the tube is shaken, placed in a boiling water bath for three minutes, and then cooled. The fluid is next filtered and 5 c.c. of the filtrate are placed into a second test tube. There are then added in succession, 0.3 c.c. of 2 N hydrochloric acid, 1 c.c. of calcium hypochloride solution,* and a pinch of calcium carbonate. The carbonate should be slightly in excess, as indicated by its persistence as a precipitate. The tube is then placed in a boiling water bath for eight minutes, after which 0.4 c.c. of 20 per cent sodium formate solution is added, and the tube is heated for eight minutes more. The tube is then removed from the bath and cooled. There are added in succession 3 c.c. of 2 N hydrochloric acid and 1 c.c. of 10 per cent potassium iodide solution. The tube is shaken and 0.5 c.c. of starch solution is added. The contents are then titrated with 0.0004 N sodium thiosulfate solution. A blank determination is made whenever new reagents are prepared: the blank ordinarily ranges between 1.0 and 1.7 c.c. of thiosulfate and should be subtracted from the volume of thiosulfate used in analysis. Each cubic centimeter of 0.0004 N thiosulfate corresponds to 7.6 mg. per 100 c.c. of bromide or 9.5 mg. per 100 c.c. of sodium bromide in the blood upon which the determination is made.

In Table IV are given the results of a series of 50 determinations made on blood (containing less than 0.05 mg. per 100 c.c. of sodium bromide) to which amounts of sodium bromide, ranging from 50 to 300 mg. per 100 c.c., had been added. The range of absolute errors is fairly constant for all concentrations of bromide and is +6 to −5 mg. per 100 c.c.; the percentage error, therefore, de-

TABLE IV

RECOVERIES OF SODIUM BROMIDE ADDED TO BLOOD

KNOWN CONCENTRATIONS OF SODIUM BROMIDE (MG./100 C.C.)	CONCENTRATION OF SODIUM BROMIDE FOUND (MG./100 C.C.)	RANGE OF ERRORS	
		MG./100 C.C.	PER CENT OF SODIUM BROMIDE PRESENT
25	22, 28, 24, 25, 28, 26, 23, 27, 24, 27	+3, −3	+12, −12
50	47, 52, 46, 48, 53, 50, 55, 46, 46, 52	+5, −4	+10, − 8
100	103, 98, 96, 104, 105, 95, 99, 97, 102, 102	+5, −5	+ 5, − 5
150	153, 147, 146, 150, 150, 156, 149, 151, 153, 149	+6, −4	+ 4, − 3
200	204, 195, 196, 202, 200, 198, 203, 199, 200, 202	+4, −4	+ 2, − 2
300	297, 299, 298, 304, 300, 301, 297, 300, 306, 303	+6, −3	+ 2, − 1

*A 15 per cent solution of $Ca(OCl)_2$ is filtered and the filtrate is diluted with water 1:3.

creased correspondingly with the increase in concentration. At 25 mg. per 100 c.c. in the blood the maximum error was 12 per cent, at 100 mg. per 100 c.c., 5 per cent, and at 300 mg. per 100 c.c., 2 per cent. Such errors are of no clinical significance, particularly as concentrations below 100 mg. per 100 c.c. rarely have clinical importance. In addition to the series of determinations shown in Table IV, analyses were made of the bromide content of the blood of 120 subjects known to have taken no bromide medicinally; the concentrations found ranged from 0 to 5 mg. per 100 c.c., values of no clinical importance. A total of more than 11,000 determinations have been made with the clinical method described and the majority in duplicate with no indications of error beyond the ranges given here.

CONCENTRATION OF BROMIDE IN WHOLE BLOOD AND SERUM

The concentration of bromide is ordinarily recorded clinically as sodium bromide in the serum. The method as described here is applied to whole blood. The analytical method is entirely applicable to serum, but the use of whole blood does away with the necessity of drawing large samples of blood. The concentration of bromide in the serum is somewhat greater than in the whole blood, since, as with chloride, there is less bromide in the corpuscles than in the serum. The difference is, however, slightly less than for chlorides[19]; the relation appears to be approximately 1:0.7. In the clinical determination of bromides even a considerable variation in the number of red blood cells would not introduce a large error. Thus with a bromide distribution of 0.7, 100 mg. per 100 c.c. of bromide in whole blood would at a hematocrit reading of 50 indicate a serum of 118 mg. per 100 c.c.; at 40, 114 mg. per 100 c.c.; at 30, 110 mg. per 100 c.c.; and at 20, 106 mg. per 100 c.c.

A series of determinations was made to relate the concentrations of bromide in serum and whole blood. One hundred and thirty-one analyses were made of blood and serum on 50 different subjects who were receiving, or had recently received, bromide and showed concentrations in the blood ranging between 25 and 190 mg. per 100 c.c. Blood was drawn by venepuncture, oxalate was used as an anticoagulant, and a specimen of the whole blood and of the serum obtained from it were analyzed for bromide. The ratio of bromide in whole blood to that in serum averaged 0.88, with extremes of 0.94 and 0.87. Thus the concentration of bromide in whole blood was approximately 12 per cent less than in serum. Conversion can be made by multiplying the results obtained from whole blood by 1.14 without introducing an error of much more than ±4 per cent. In the extreme instance as that calculated above from the assumed distribution between serum and corpuscles in marked anemia (hematocrit reading 20), the error would be increased to approximately 8 per cent.

CONCLUSIONS

1. The principles of determining bromides in biological materials are discussed critically.

2. A rapid and accurate method is described for determining both bromides and chlorides on a single sample of blood or tissue. Acid digestion is used and the silver halides are converted to soluble halides by treatment with thiosulfate.

3. A rapid and reliable clinical method is described by which bromide can be determined directly in 0.1 c.c. of blood drawn from a needle prick. The method is more accurate than any clinical method previously described and avoids the necessity for venepuncture.

REFERENCES

1. Neufield, A. H.: Contributions to the Biochemistry of Bromine I, Canad. J. Research 14: 160, 1936.
2. Brodie, B., and Friedman, M.: Determination of Bromide in Tissues and Biological Fluids, J. Biol. Chem. 124: 511, 1938.
3. Kendall, E. C.: Determination of Iodine in Connection With Studies in Thyroid Activity, J. Biol. Chem. 43: 149, 1920.
4. Carius, L.: Bestimmung von Schwefel, Chlor, Phosphor u. s. w. in organischen Substanzen, Ann. d. Chem. u. Pharm. 136: 129, 1865.
5. Hartner, F.: Determination of the Bromide Content of Blood, Ztschr. f. anal. Chem. 25: 195, 1934.
6. Doering, H.: Zur titrimetrischen Bestimmung Kleinster Brommenger bei Gegenwart von Chloriden, Ztschr. f. anal. Chem. 108: 255, 1937.
7. Labat, A.: Sur la presence du brome à l'état normal dans les organes de l'homme, Compt. rend. Acad. d. Sc., Paris 156: 255, 1913.
8. Deniges, G., and Chelle, L.: Nouveau réactif du chlore et du brome libres et combinés, Compt. rend. Soc. de biol. 155: 1010, 1912.
9. Walter, F.: Untersuchungen über die Permeabilität der Meningen, Ztschr. f. d. ges. Neurol. u. Psychiat. 99: 380, 1925.
10. Wuth, O.: Rational Bromide Treatment; New Methods for Its Control, J. A. M. A. 88: 2013, 1927.
11. Diethelm, O.: On Bromide Intoxication, J. Nerv. & Ment. Dis. 71: 151, 1930.
12. Francis, A., and Harvey, C.: Micro-determination of Bromine in Blood, Biochem. J. 27: 1545, 1933.
13. Bertram, S. H.: Die Bestimmung kleiner Brommengen neben grösserer Mengen Chlor und Jod, Biochem. Ztschr. 261: 202, 1933.
14. Yates, E.: Determination of Bromine in Blood, Biochem. J. 27: 1763, 1933.
15. van der Meulen, J. H.: Bromoiodometric Investigations. V. Determination of Bromine in Bromides, Chem. Weekbl. 28: 82, 1931; Bromoiodometric Investigations. VI. Determination of Bromides, Chem. Weekbl. 28: 238, 1931.
16. Kolthoff, I. M., and Yutzy, H.: Volumetric Determination of Bromide After Oxidation to Bromate in the Presence of Much Chloride, Indust. & Engin. Chem. (Anal. Ed.) 9: 75, 1937.
17. Dixon, T. F.: Determination of Bromine in Normal Blood, Biochem. J. 28: 48, 1934.
18. Volhard, J.: Die Silbertitrirung mit Schwefelcyanammonium, Ztschr. f. anal. Chem. 17: 482, 1878.
19. Mason, M. F.: Halide Distribution in Body Fluids in Chronic Bromide Intoxication, J. Biol. Chem. 113: 61, 1936.

THE DETERMINATION OF DEMEROL IN URINE WITH PRELIMINARY OBSERVATIONS ON ITS EXCRETION IN MAN*

ROBERT A. LEHMAN, PH.D., AND THEIS AITKEN, M.S.
NEW YORK, N. Y.

CONSIDERABLE interest has arisen concerning the analgesic and antispasmodic properties of demerol which is the hydrochloride of 1-methyl-4-phenyl-piperidine-4-carboxylic acid ethyl ester. A number of reports have already appeared concerning its action on various physiologic functions, both in animals and in man.[1-8] To facilitate the study of the fate and manner of disposal of demerol in the animal body a method is suggested for determining demerol in urine, and some preliminary data are included on the urinary elimination of the drug in man.

The method depends upon the formation of a benzene-soluble compound between the free base of demerol and the dye bromthymol blue (dibromthymolsulfonphthalein) in an equimolar ratio. The free base is first extracted from the urine with benzene at pH 7.5. The resulting benzene solution of demerol is then shaken with an aqueous solution of the dye, also buffered at pH 7.5, whereupon an amount of dye approximately equivalent to the amount of demerol is carried into the benzene as a yellow compound.† This compound is readily decomposed so that the sodium salt of bromthymol blue may be extracted by aqueous alkali while the free base of demerol remains in the benzene and is discarded. The amount of dye extracted may be determined colorimetrically, and thus, indirectly, the original concentration of demerol.

PROCEDURE

The following solutions are required:

1. Concentrated buffer solution: 3.68 Gm. of $NaH_2PO_4 \cdot H_2O$ and 38.2 Gm. of $Na_2HPO_4 \cdot 12\ H_2O$ are dissolved in distilled water and made up to one liter.

2. Dilute buffer solution: 250 c.c. of solution 1 is diluted with an equal volume of distilled water.

3. Stock bromthymol blue solution: 0.400 Gm. of bromthymol blue (Eastman) is dissolved with warming in 30 c.c. of water and 6.4 c.c. of 0.100 N sodium hydroxide and made up to 100 c.c.

4. Dilute bromthymol blue solution: Solution 3 is diluted 1:5 with distilled water.

5. Buffered bromthymol blue solution: 40.0 c.c. of solution 4 are made up to 100 c.c. with solution 2.

The first operation is to buffer the urine to approximately pH 7.5. For this purpose a preliminary test is made. A drop of the stock bromthymol blue solu-

*From the Department of Therapeutics, New York University College of Medicine.
Received for publication, June 29, 1942.

†We are indebted to Dr. O. W. Barlow and Dr. D. R. Climenko for suggesting the application of this principle to the determination of demerol.

tion is added to a mixture of 10 c.c. of concentrated buffer solution and 10 c.c. of the urine to be analyzed. The color of the resulting solution should be greenish blue to blue. If it is yellowish green to green, about 0.5 N sodium hydroxide solution is added drop-wise from a burette until the greenish-blue shade is produced. The indicated amount of sodium hydroxide is later added, in addition to the buffer, to fresh 10 c.c. aliquots of urine for analysis. If the urine contains a suspended precipitate, as is often the case, the test mixture should be centrifuged and the color of the supernatant liquid observed.

Three 250 c.c. separatory funnels are required for each determination. Ten cubic centimeters of concentrated buffer solution, 10 c.c. of the urine to be analyzed, and the proper amount of 0.5 N sodium hydroxide, as determined by the preliminary test, are placed in the first funnel, and enough water is added to bring the volume to 25 c.c. The demerol is extracted from the aqueous layer by shaking for thirty seconds with 60 c.c. of benzene. The aqueous layer is drained into the second funnel while the yellow benzene layer is decanted through the neck into the third funnel which must be dry.* The extraction is repeated twice with 50 c.c. portions of benzene, which are added to the first extract. The combined extracts are then shaken for two minutes with 25 c.c. of buffered bromthymol blue solution. The aqueous layer is drained into a flask and the benzene layer is decanted into a dry Erlenmeyer flask. The bromthymol blue solution is returned to the same funnel and is shaken gently with 35 c.c. of fresh benzene. Too vigorous shaking will result in an emulsion which separates only after long standing. The aqueous layer is now discarded, and the benzene extract is combined with the main portion which is allowed to stand until crystal clear. One to two hours is usually sufficient. The combined benzene extract is now decanted into a clean separatory funnel and extracted twice with 40 c.c. portions of 0.05 N sodium hydroxide solution, and the combined alkaline extracts are made up to 100 c.c. with 0.05 N sodium hydroxide. However, if the amount of demerol present in the original 10 c.c. of urine did not exceed 0.1 mg., it is preferable to extract the benzene solution with two 20 c.c. portions of 0.05 N sodium hydroxide and make the combined extracts up to 50 c.c. With practice, one can readily tell from the depth of yellow color in the benzene whether or not the amount of demerol in the original 10 c.c. of urine was greater than 0.1 mg.

The percentage transmission of the blue solutions is determined in a photoelectric colorimeter using a Corning filter combination of No. 350 (H.R. Traffic Yellow) and No. 398 (Dark Aklo).† The amount of demerol in the original 10 c.c. of urine is then determined by reference to a curve (Fig. 1) relating the log percentage transmission of the final blue solutions to the milligrams of demerol per 10 c.c. of urine. This curve was obtained by analyzing 10 c.c. aqueous samples containing appropriate amounts of demerol in place of urine in ex-

*In order to obtain benzene solutions practically free of the aqueous solution, each separation throughout the procedure is made as follows: the aqueous layer is drained through the stem of the funnel, and the funnel is rotated briskly to bring about a further separation of aqueous solution which, together with the major portion of any emulsion formed at the interface, is also drained through the stem. The benzene is then decanted through the neck of the funnel in such a way that traces of emulsion clinging to the sides of the funnel are retained on the walls.

†The absorption maximum of an alkaline bromthymol blue solution occurs at 6,170 angstrom units. This filter combination cuts off sharply at 5,400 angstrom units. Other combinations of filters were tried but none gave a steeper or more nearly linear curve of Beer's law.

actly the procedure described above. It will be seen from Fig. 1 that the curve follows the Lambert-Beer law in the range zero to 0.400 mg. of demerol, and deviates only slightly over the rest of the range.

It would appear that a curve based directly upon standard solutions of bromthymol blue might be used rather than the empirical curve (Fig. 1) for which each point must be obtained by the complete extraction process. This is not the case, however, because the theoretical amounts of bromthymol blue are obtained only approximately in the extraction process. The per cent of the actual amount of bromthymol blue obtained in the final extract to the theoretical amount expected was found to change progressively with the demerol content

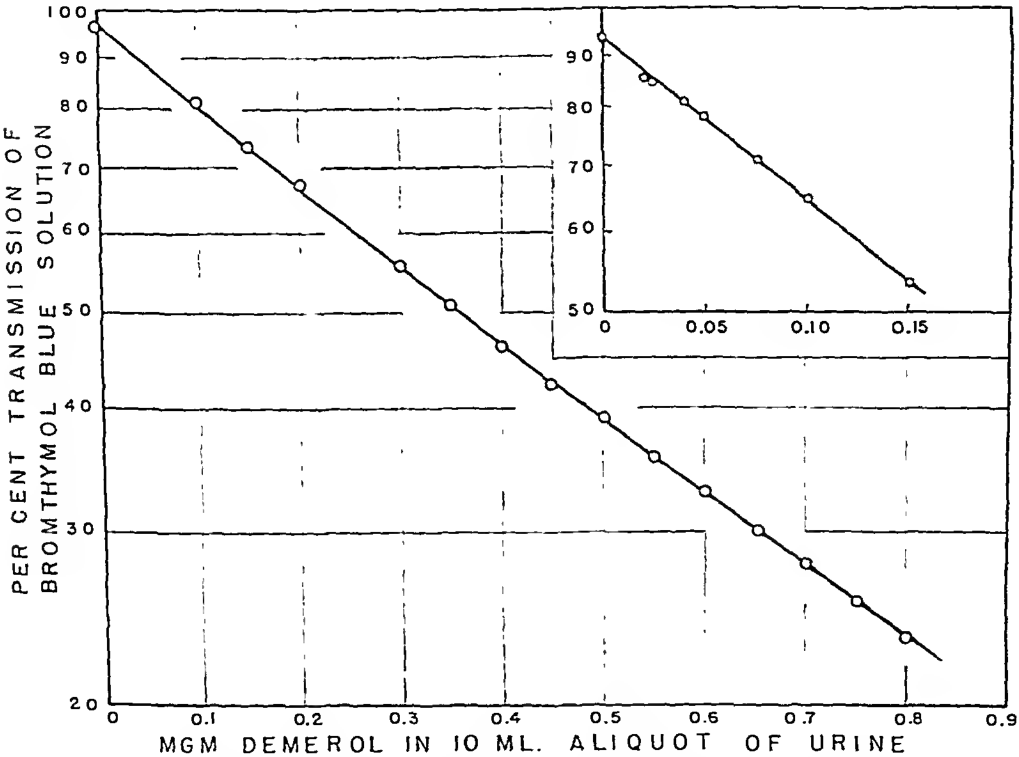


Fig. 1.—Relationship between the percentage transmission of bromthymol blue solution and the amount of demerol present in the original 10 c.c. sample of urine or aqueous solution. The inset shows the same relationship in the case where the final bromthymol blue solution has been diluted to 50 c.c. instead of 100 c.c.

of the original aqueous solution, as shown in Table I. The probable reasons for this deviation from theoretical are: (1) incomplete extraction of demerol base in the first step of the procedure; (2) loss of demerol into the aqueous layer in the second step, this being governed by the concentration of the excess dye, which, of course, is dependent upon the concentration of demerol; (3) extraction by the benzene layer of some uncombined dye present as a result of hydrolysis of sodium bromthymol blue in the buffered solution, the amount, which is small, being directly proportional to the amount of dye and indirectly proportional to the amount of demerol; and (4) the mechanical transfer of dye in the last step of the procedure. The curve does not pass through the origin, due to reasons (3) and (4).

TABLE I

VARIATION IN THE PER CENT OF A MOLAR EQUIVALENT OF BROMTHYMOL BLUE FOUND FOR DIFFERENT CONCENTRATIONS OF DEMEROL

CONCENTRATION OF DEMEROL TAKEN (MG./10 C.C.)	PER CENT OF A MOLAR EQUIVALENT OF BROMTHYMOL BLUE FOUND IN FINAL EXTRACT
0.05	120
0.10	100
0.15	97
0.20	93
0.25	92
0.30	92
0.40	89
0.50	88
0.60	86
0.70	87
0.80	86

TABLE II

RECOVERY OF DEMEROL FROM AQUEOUS SOLUTIONS

Analysis of 10 c.c. samples

DEMEROL TAKEN (MG.)	DEMEROL FOUND (MG.)	PER CENT ERROR
0.700	0.694	-0.9
0.700	0.699	-0.1
0.680	0.684	-0.6
0.650	0.651	+0.2
0.600	0.600	0
0.580	0.577	-0.5
0.550	0.550	0
0.500	0.503	+0.6
0.500	0.505	+1.0
0.450	0.451	+0.2
0.400	0.398	-0.5
0.350	0.351	-0.3
0.300	0.300	0
0.250	0.248	-0.8
0.200	0.201	+0.5
0.150	0.150	0
0.100	0.099	-1.0
0.075	0.072	-4.0
0.065	0.064	-1.5
0.060	0.057	-5.0
0.050	0.049	-2.0

RESULTS

Recoveries of demerol from aqueous solutions are shown in Table II. When the procedure was first applied to urine, it was noted that fresh alkaline urines (twenty-four-hour specimens) have a considerable blank, or apparent demerol concentration, while fresh acid urines have little or none. Old urines which have become alkaline on standing also show a blank. Since blanks occur in alkaline urines and not to any great extent in acid urines, it seems likely that the blanks are due to basic decomposition products. In order to study this factor a urine containing a known amount of demerol was aged under various conditions to show the effect of inhibiting decomposition with mercuric chloride, of allowing decomposition to occur normally, and of facilitating it by making the urine alkaline. Table III shows the results of this study. The demerol content of the portion of urine preserved with mercuric chloride did not change during the ten-day aging period, while the untreated portion had an apparent demerol

concentration of nearly twice the original value, and the portion made alkaline showed more than twice the original value.

Many organic bases react similarly to demerol, and the presence of such compounds in the urine will give rise to a blank or apparent demerol content. Quinine, codeine, aminopyrine, ephedrine, and nicotine have a demerol-like reaction, and it is likely that many other alkaloids do also. Morphine, however, in amounts likely to be present in the urine does not interfere seriously with the de-

TABLE III

EFFECT OF AGING UNDER VARIOUS CONDITIONS ON THE RECOVERY OF DEMEROL FROM URINE
Analysis of 10 c.c. samples containing 0.128 mg.

TREATMENT	DEMEROL FOUND (MG.)
Analyzed immediately	0.133 0.135
0.5 Gm. $HgCl_2$ added, analyzed after ten days	0.134 0.134
No treatment, analyzed after ten days	0.239 0.239
Made alkaline to litmus with solid $NaHCO_3$, and analyzed after ten days	0.283 0.292

TABLE IV

RECOVERIES OF DEMEROL ADDED TO URINES COLLECTED WITH MERCURIC CHLORIDE FROM SUBJECTS RECEIVING NO DRUGS

Analysis of 10 c.c. samples.

SUBJECT	DEMEROL TAKEN (MG.)	DEMEROL FOUND (MG.)	PER CENT ERROR
1	0.084	0.085	+1.2
	0.084	0.083	−1.2
2	0.113	0.114	+0.9
	0.113	0.114	+0.9
3	0.120	0.132	+10.0
	0.120	0.130	+8.3
4	0.400	0.400	0
	0.200	0.206	+3.0
5	0.400	0.401	+0.3
	0.200	0.194	−3.0
6	0.100	0.100	0
	0.325	0.325	0
7	0.093	0.096	+3.2
	0.093	0.094	+1.1
8	0.094	0.099	+5.3
	0.094	0.096	+2.1
9	0.300	0.324	+8.0
	0.300	0.327	+9.0
10	0.218	0.227	+4.1
	0.218	0.226	+3.7
11	0.134	0.133	−0.7
	0.134	0.137	+2.2
12	0.185	0.183	−1.1
	0.185	0.185	0
13	0.144	0.145	+0.7
	0.144	0.146	+1.4

termination of demerol, since 10 mg. of morphine sulfate give an apparent demerol concentration of only about 0.1 mg.

Table IV shows recoveries of demerol added to normal twenty-four-hour urines which were collected in bottles containing 20 c.c. of saturated mercuric chloride solution from 13 subjects receiving no drugs.

In order to determine the order of magnitude of the excretion of demerol, the drug in total doses of 100 to 200 mg. daily was administered to three subjects, and the twenty-four-hour urine specimens were analyzed by the method just described, using mercuric chloride as a preservative. The results are shown in Fig. 2. The data have been plotted in terms of per cent of the total administered demerol which had been recovered in the urine at any given time. It will

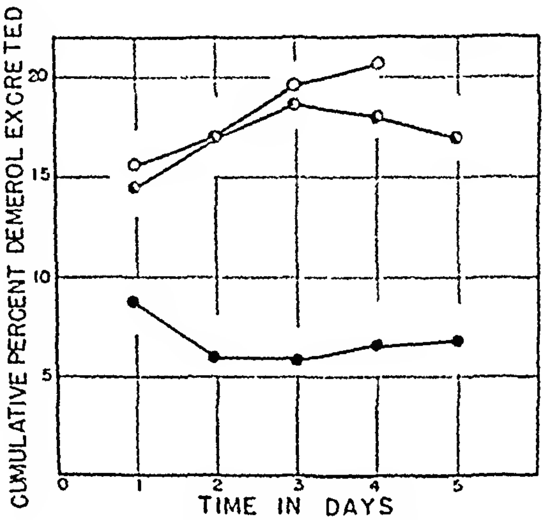


Fig. 2.—Excretion of demerol after oral administration of 100 to 200 mg. daily.

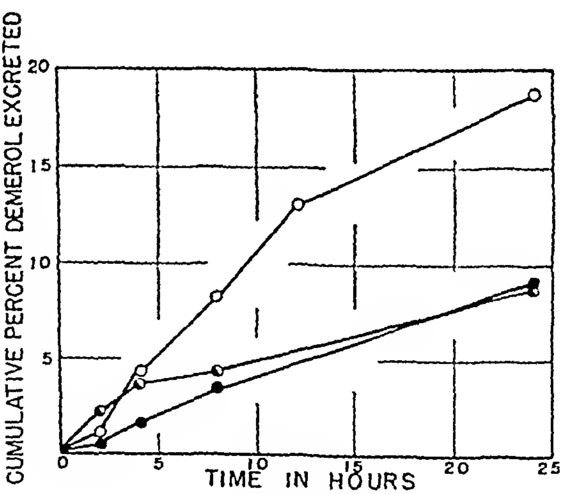


Fig. 3.—Excretion of demerol after subcutaneous injection of 100 mg.

be seen that, roughly, 5 to 10 per cent of the demerol is eliminated daily. To test the possibility that the demerol remaining unaccounted for may have escaped absorption in the intestinal tract, 100 mg. were administered subcutaneously to each of three subjects, and the urine was analyzed at intervals for the first twenty-four hours. The results of this study are shown in Fig. 3, and they agree in order of magnitude with those in Fig. 2. Thus, of 100 mg. injected, about 5 to 20 per cent has been excreted at the end of twenty-four hours. From

the agreement between the two routes of administration it seems probable that incomplete absorption is not accountable for the demerol which fails to appear in the urine.

SUMMARY

A method has been described by which demerol can be determined in amounts from 0.05 to 0.7 mg. per 10 c.c. of aqueous solution, with an average error of 0.9 per cent. The recoveries from urine indicate that amounts of demerol between 0.08 and 0.4 mg. per 10 c.c. (8 to 40 mg. per liter) can be determined, with an average error of 2.7 per cent. In no case did the error exceed 10 per cent.

Basic products resulting from the aging of urine cause a blank or apparent demerol concentration. Mercuric chloride prevents the formation of such products, and it is essential that it be used routinely in the collection of specimens.

The presence of various alkaloids in the urine interferes with the determination of demerol and care should be exercised accordingly in analyzing specimens when the subject has received other medication. Morphine does not interfere.

Preliminary excretion studies have been reported on six subjects who received therapeutic doses of demerol.

REFERENCES

1. Batterman, R. C.: The Clinical Effectiveness and Safety of a New Synthetic Analgesic Drug, Demerol, Arch. Int. Med. In press.
2. Ibid: Demerol—A New Analgesic Drug Effective for Relief of Pain in Medical and Surgical Conditions, Tr. Am. Therap. Soc. In press.
3. Batterman, R. C., and Mulholland, J. H.: Demerol, a Substitute for Morphine in the Treatment of Postoperative Pain, Arch. Surg. In press.
4. Duguid, A. M. E., and Heathcote, R. St. A.: Pharmacological Action of Ethylmethylphenylpiperidinecarboxylate, Quart. J. Pharm. & Pharmacol. 13: 318, 1940.
5. Eisleb, O., and Schaumann, O.: Dolantin, ein neuartiges Spasmolytikum und Analgetikum (Chemisches und Pharmakologisches), Deutsche med. Wchnschr. 65: 967, 1939.
6. Gruber, C. M., Hart, E. R., and Gruber, Jr., C. M.: The Pharmacology and Toxicology of the Ethyl Ester of 1-Methyl-4-phenyl-piperidine-4-carboxylic Acid (Demerol), J. Pharmacol. & Exper. Therap. 73: 319, 1941.
7. Himmelsbach, C. K.: Studies of the Addiction Liability of Demerol (D-140), J. Pharmacol. & Exper. Therap. 75: 64, 1942.
8. Rovenstine, E. A., and Batterman, R. C.: The Utility of Demerol as a Substitute for the Opiates in Preanesthetic Medication, Anesthesiology. In press.

BOOK NOTICES

Anoxia*

DR. VAN LIERE who has contributed a great deal to the subject of anoxia writes with justified authority. His book is primarily a discussion of the altered physiology of anoxia as it affects blood, tissues, cells, and the various body systems. There are relatively short chapters on mountain sickness and altitude sickness and acclimatization.

The volume will be of especial interest in aviation medicine, physiology, and biochemistry, and a helpful reference work for the clinician.

The Lymphatic System†

THE LYMPHATIC SYSTEM is a series of five lectures given at Stanford University in 1941. Dr. Drinker's first lecture discusses the evolution of the mammalian circulation. His second lecture is on the development and characteristics of the capillary circulation. The third lecture deals primarily with the lymphatic system. In the fourth, he discusses normal and abnormal physiology of the body fluids. The final lecture deals with practical problems in medicine and surgery.

Written by a world authority on the lymphatic system, this is essentially a practical summary which should be of value both to laboratory investigators and clinicians.

The Doctors Mayo‡

IT WAS to be anticipated that a biography of the Mayos, father and sons, would appear reasonably soon after the death of the last of this famous trio. These men represented the greatest achievement of private group practice that the world will probably ever see. While group practice will continue, it will undoubtedly be taken over increasingly by the state as a public service.

The contents of this book are sponsored by the University of Minnesota and are authentic. The volume is a little long for easy reading, but the most interesting part is the first half which describes the earlier formative years of the Mayo Clinic. The latter part of the book can be skimmed, and with considerable profit to the reader.

Manual of Clinical Chemistry§

REINER'S *Manual of Clinical Chemistry* is a small "pocket" book containing adequate directions for performing nearly all the clinical chemical examinations that are likely to be called for in hospital laboratories. Included are quantitative tests for the various hormones and vitamins; also toxicological tests, arsenic, lead, mercury, and barbiturates. There is one error of a decimal point on page 5 which, however, does not invalidate any of the procedures described in the book.

*Anoxia. Its Effect on the Body. By Edward J. Van Liere, Ph.D., M.D. Cloth, 269 pages, $3.00. The University of Chicago Press, Chicago, Ill., 1942.

†La[illegible] The Lymphatic System. Its Part in Regulating Composition and Volu[illegible] By Cecil K. Drinker, Professor of [illegible] of the School of [illegible]vard University. Stanford Univers[illegible] iversity Series, M[illegible] V, No. 2. Cloth, 101 pages, $2.25. [illegible] Press, Stanford [illegible] 1942.

‡The Doctors Mayo. By Helen Clapesattle. Cloth, 822 pages, $3.75. The University of Minnesota Press, Minneapolis, 1941.

§Manual of Clinical Chemistry. By Miriam Reiner, M. Sc., Assistant Chemist to the Mt. Sinai Hospital, New York. Introduction by Harry Sobotka, Ph.D., Chemist to the Mt. Sinai Hospital, New York. Cloth, 296 pages, with 18 illustrations, $3.00. Interscience Publishers, Inc., New York, 1941.

Neuroanatomy*

DR. METTLER'S book is primarily a student text. The first portion deals with gross anatomy; the second, with microscopic anatomy. The book is so organized that when the medical student who has used it in his first year anatomy reaches his third and fourth year work in clinical neurology, he will find it a very handy reference volume. It is abundantly and well illustrated.

Pediatric Gynecology†

SIXTEEN years ago Dr. Schauffler, faced with a clinical problem of disease involving the genitals in a female child, found to his surprise that discussions of the general subject in the medical literature were few and rather noninformative. The present volume presents the results of his studies in the ensuing years. It is entirely a clinical book. It deals with methods of examination and treatment of disease of the sexual organs and other pelvic pathology in female children. The discussion carries on through abnormalities of adolescence. Diseases discussed include vaginitis, pelvic peritonitis, enuresis, pyelitis, rectal pathology, dysmenorrhea, and a large number of other conditions. There are sections on social service aspects of the treatment and supervision of these children; also on medicolegal aspects, in which is included a discussion of rape. The appendix includes lists of standard hormone preparations.

The work is practical and authoritative.

The Physiology of Domestic Animals‡

THE Physiology of Domestic Animals will be of greater use to veterinary physicians than to those dealing with human disease. However, it will be of distinct value to those who are interested in comparative physiology and to those in the field of experimental medicine who deal with animals in their work.

Methods of Treatment§

IT WOULD be rather difficult to furnish "an outline of all the methods of treatment in internal medicine" in a single volume of less than a thousand pages. Dr. Clendening has gone far toward accomplishing this. In succeeding editions the coverage has been increasingly complete. Naturally, the space devoted to any one subject is decidedly limited and the treatment is at times superficial. However, the volume serves very nicely as an index to treatment and as such makes a very adequate quick reference volume.

---

*Neuroanatomy. By Fred A. Mettler, A.M., M.D., Ph.D., Professor of Anatomy, University of Georgia School of Medicine, Augusta, Ga. Cloth, 476 pages, with 337 illustrations, including 30 in color. The C. V. Mosby Company, St. Louis, 1942.

†Pediatric Gynecology. By Goodrich C. Schauffler, A.B., M.D., Assistant Clinical Professor of Obstetrics and Gynecology, University of Oregon Medical School; Visiting Gynecological Surgeon and Obstetrician, Multnomah Hospital, Portland, Ore. Cloth, 384 pages, $5.00. The Year Book Publishers, Inc., Chicago, Ill., 1942.

‡The Physiology of Domestic Animals. By H. H. Dukes, D.V.M., M.S., Professor of Veterinary Physiology, New York State Veterinary College, Cornell University. With a chapter on the Physico-Chemical Basis of Physiological Phenomena by E. A. Hewitt, D.V.M., Ph.D., Associate Professor of Veterinary Physiology, Division of Veterinary Medicine, Iowa State College; a Part on the Endocrine Organs and Reproduction, revised by S. A. Asdell, M.A., Ph.D., Professor of Animal Physiology, New York State College of Agriculture, Cornell University; and a Foreword by H. D. Bergman, D.V.M., Professor of Veterinary Physiology and Pharmacology, Division of Veterinary Medicine, Iowa State College. Cloth, ed. 5, revised, 721 pages, $6.00. Comstock Publishing Company, Inc., Ithaca and New York, 1942.

§Methods of Treatment. By Logan Clendening, M.D., Clinical Professor of Medicine, Medical Department of the University of Kansas; Attending Physician, University of Kansas Hospitals; and Edward H. Hashinger, A.B., M.D., Clinical Professor of Medicine, Medical Department of the University of Kansas; Attending Physician, University of Kansas Hospitals; Attending Physician, St. Luke's Hospital, Kansas City, Mo. With Chapters on Special Subjects by J. B. Cowherd, M.D.; Leland F. Glaser, M.D.; Thomas B. Hall, M.D.; John S. Knight, M.D.; H. P. Kuhn, M.D.; Paul H. Lorhan, M.D.; F. C. Neff, M.D.; Don Carlos Peete, M.D.; Carl O. Rickter, M.D.; E. H. Skinner, M.D.; O. R. Withers, M.D.; and Lawrence E. Wood, M.D. Cloth, ed. 7, 997 pages, The C. V. Mosby Company, St. Louis, 1941.

Disturbance of the Blood Coagulation in Infants*

THE well-known Swiss pediatrician, Fanconi, reports and discusses the carefully taken history of 47 infants suffering from various disturbances of the blood coagulation mechanism. Coagulation and bleeding time, prothrombin time, fibrinogen content, heparin content, calcium content, differential blood count, platelet count, etc., were determined by the most modern methods throughout the whole course of clinical observation. The cases are grouped under five main classifications: lack of fibrinogen, lack of calcium, lack of thrombokinase, hyperproduction of heparin, lack of vitamin K. The latter group is by far the largest. However, Fanconi emphasizes that most hemorrhagic diseases of the newborn are not caused by a single factor, but result from the interaction of various factors. Each time a new scientific discovery is made, its importance is overestimated. Thus, in consequence of the good results with vitamin K therapy, the K-avitaminose has been overrated as the sole cause of most hemorrhagic diseases in infants. An extensive bibliography increases the value of this small but very interesting book.

Ernst Fischer

Blood Disorders in Children†

WELL known, not only as a pediatrician, but also as an investigator, Dr. Kugelmass in this volume presents an authoritative and comprehensive discussion of a subject of importance, not only to the pediatrician, hematologist, and clinical pathologist, but also to the clinician at large.

The arrangement of the book follows the clinical rather than the hematologic viewpoint, and thus evaluates the blood picture in terms of the patient as a whole. A blood glossary and index of differential diagnosis based on both clinical picture and blood findings greatly enhance the usefulness of the book.

An outstanding contribution of great and general interest.

ITEM

The Laboratory of Mt. Sinai Hospital, Cleveland, Ohio, is making blood studies in suspected and known cases of trichinosis and shall appreciate it if readers of THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE send whole clotted blood samples in such cases to Dr. B. S. Kline of that laboratory.

---

*[illegible] Blood Coagulation in Infants With Special Reference to the Vitamin K and [illegible] the Newborn (Die Störungen der Blutgerinnung beim Kinde mit besonde[illegible] des K-Vitamins und der Neugeborenenpathologie.) By G. Fanconi. 160 pag[illegible] Thieme, Leipzig, 1941.

†[illegible] Children. By I. Newton Kugelmass, M.D., Ph.D., Sc.D., Spec. Attending [illegible]town Hospital and Pan-American Clinics; Consultant Pediatrician, Hecksc[illegible]ild Health, New York City Children's Hospital; Lynn Memorial Hospita[illegible]rial Hospital, Muhlenberg Hospital; former Director of Pediatric Researc[illegible]d Research Associate in Pediatrics, Yale University. Cloth, 897 pages, [illegible] $10.00. Oxford University Press, New York.

The Journal of Laboratory and Clinical Medicine

Vol. 28 | April, 1943 | No. 7

CLINICAL AND EXPERIMENTAL

THE HEART IN EXPERIMENTAL THYROTOXICOSIS*

Ernest D. Nora, M.D., and
Nathan Flaxman, M.D., Chicago, Ill.

THERE are three entirely opposite schools of thought concerning the effects of thyroid disease on the heart. One group is of the opinion that thyrotoxicosis has no effect on the heart except to increase the metabolic activity.[1] Another group is inclined toward the view that the thyrotoxic disease causes myocardial damage, hypertrophy of the heart, and various clinical cardiac manifestations.[2] A third group, championed by more recent work, is of the opinion that the thyrotoxicosis acts only as a catalytic agent and brings to the surface latent cardiovascular lesions.[3]

Opinions vary not only as to the specificity of the possible thyrotoxic cardiac changes, but also as to the probable cause of such alterations in the heart. Some hold the primary cause as the toxicosis, while others attribute any cardiac changes to an indirect cause, such as the increased heart rate. The third group, as mentioned, attributes any possible changes to previous heart disease, especially a rheumatic myocarditis.

Thyrotoxicosis, experimentally induced, has not been entirely successful in solving the problem of the cardiac lesions in hyperthyroidism. Specific lesions have been produced in various animals.[4] However, others have reported that they were unable to demonstrate specific lesions in the hearts of experimental animals.[5]

Neither the clinical nor the experimental data at hand are sufficient to enlighten the problem of the effects of the thyroid upon the heart muscle in hyperthyroidism. In an experimental study somewhat similarly performed, Menne et al.,[5] found certain cardiac changes in the hearts of rabbits that were

*From the Columbus Hospital and the Department of Medicine, Loyola University School of Medicine.

Received for publication, August 15, 1942.

fed desiccated thyroid, but they concluded that it was possible that similar changes might be produced by cardiac overwork irrespective of the presence of thyroxin in the circulating blood. In an effort to investigate further this important and complex problem, the following experiments were undertaken.

METHODS OF INVESTIGATION

The work was performed entirely on rabbits. The animals were all of average weight and age. They were housed in a warm room and fed a normal rabbit diet, sufficient in quantity to ensure normal maintenance in weight which was determined by periodic weighings every four or five days.

Electrocardiographic tracings with Lead II were taken to determine accurately the pulse rate. Needle contacts were not employed, but a method with Sanborn Redux Paste was used. The hair on the areas of the right foreleg and left hindleg needed for contact were shaved and the paste rubbed thereon. Eight strand copper wire electrodes were employed, and these were wound twice around the selected area on the limb and again covered with the paste. Being painless, the method did not excite the animals, and tracings were obtained rapidly.

A normal electrocardiogram was obtained from each animal after it had become accustomed to being tied down on the animal board. Tracings were taken about every seven days, or thereafter, or until the death of the animal.

They were divided into six experimental groups, as follows: Group I, rabbits in which only the cardiac inhibitory nerves were removed; Group II, rabbits in which the cardiac inhibitory nerves were removed and digifoline (Ciba) injected subcutaneously; Group III, rabbits in which the cardiac inhibition was removed and quinidine sulfate administered orally; Group IV, desiccated thyroid (Armour) fed rabbits; Group V, thyroid fed and digifoline injected rabbits; and Group VI, thyroid and quinidine fed rabbits.

In the first three groups, where the cardiac inhibitory nerves were removed, it was performed in the following manner. The animals were given ether anesthesia, and a midline incision in the neck exposed the carotid sheaths. The pressor nerve of the vagus was isolated on each side, and a 1 to 2 cm. section was removed. The carotid arteries were stripped of their coverings up as far as the bifurcation into the internal and external branches and then swabbed with absolute alcohol. The animals were allowed to recover in a warmed room for several days.

Animals of one litter were used in the last three groups to exclude, as well as possible, any previous cardiac changes. The animals were all housed and fed similarly, and, in addition, were fed desiccated thyroid to produce toxicosis. The amount of thyroid fed varied with each individual animal, some becoming toxic on 2-3 grains per day and others requiring as much as 8 to 16 grains per day. They were fed the thyroid until the symptoms of toxicosis were manifested by weakness, diarrhea, loss of weight, prostration, lethargy, and increased pulse. Some of the animals, those that survived, were then given a rest period, and when the pulse and weight returned to normal, a second toxicosis was induced. This was continued until the animal died, or a sixty-day observation period passed, when the animal was killed. Normal electrocardio-

TABLE I

GROUP I. EXPERIMENTAL DATA CONCERNING RABBITS WITH CARDIAC INHIBITION REMOVED

								LEFT VENTRICLE						RIGHT VENTRICLE						
ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS	PULSE CHANGE	DEATH	HEART WEIGHT	LENGTH	ABOVE	BELOW	RING	DIAM.	CIRC.	LENGTH	ABOVE	BELOW	DIAM.	CIRC.	ECG	COURSE
3	2.65	60	210	0	+30	K	9	22	6	4	12	14	28	22	1.5	1	14	28	Normal	Negative
1	2.8	60	250	0	+15	K	10	25	8	6	14	18	27	25	3	2	18	27	Normal	Negative
6	2.15	60	230	0	+10	K	10	29	8	6	12	14	29	29	2	1.5	14	29	Normal	Negative
7	2.62	60	210	0	+20	K	9	29	6	4	11	14	27	29	2	1	14	26	Normal	Negative
2	2.0	1	240	0	0	D	8.2	21	6	5	12	15	27	23	2.5	1.5	15	27	Normal	Carotid punctured
8	2.3	1	210	0	0	D	8	23	4	3	12	13	25	23	1.5	1.0	13	26	Normal	Vagus cut
9	3.0	3	240	0	0	D	12	27	6	3	14	16	29	27	1.5	1.0	16	30	Normal	Inf. Laryngeal cut
11	2.9	1	210	0	0	D	10	25	6	5	12	15	30	25	3	2	15	28	Normal	Vagus cut
16	2.4	7	210	0	+70	D	9	22	6	4	12	18	30	23	3.5	2	18	32	T waves negative	Suppurative pericarditis
18	2.48	0	170	0	0	D	8.7	20	6	5	11	16	29	20	3	1.5	16	29	Normal	Death due to anesthesia
23	2.92	0	160	0	0	D	9	23	6	4	13	20	36	23	2	1	20	25	Normal	Laryngeal obstruction

Heart weight is in grams.
All measurements are in millimeters.

graphic tracings were obtained as described, and tracings were taken every seven days thereafter.

Group I. Cardiac Inhibition Removed.—This group consisted of 11 rabbits that were operated in the manner described. Seven of the 11 animals died in one to seven days due to pulmonary complications, apparently resulting from the severing of other vagal branches besides the pressor nerve. Four animals survived and were killed at the end of the observation period. All were autopsied to exclude other conditions which might cause any cardiac pathology (Table I).

Although there were minor variations in certain cases, gross examination of the hearts in this group revealed that the muscle, in general, was moderately firm and the auricular chambers dilated. Epicardial fat was scant, and the ventricles appeared hypertrophic. The hearts of the seven animals found dead had dilated cavities filled with blood clots. The average weight of the hearts of the animals killed at the end of sixty days was 9.5 grams, and of those found dead, 9.4 grams. There was a difference in the thickness of the left ventricular musculature. In the animals killed it measured 7 mm. above and 5 mm. below, while in the animals found dead it was 5.7 mm. above and 4.3 mm. below. The length of the ventricles from the opening of the coronary artery to the tip of the apex was 3.1 cm. in the killed and 2.7 cm. in the animals found dead. The ventricular diameter was the same in both sets of rabbits, 1.6 cm.

For the microscopic examination three different sections were taken from all the hearts. One section was through the left ventricle, a second through the right ventricle, and the third section through a papillary muscle. In the animals that died shortly after operation no microscopic changes were noted in the heart muscle. In Rabbit No. 16, where the autopsy revealed a stitch abscess with penetration of the infection into the mediastinum and a resultant fibropurulent pericarditis, the microscopic examination of the heart showed hyalinization and separation of the muscle bundles with round and neutrophilic cellular exudation. In the four animals that survived the operation and were observed for sixty days, the findings were uniformly positive. These hearts showed hyalinization and granular degeneration with slight to moderate fragmentation and separation of the muscle bundles. Lymphocytic infiltration was noted in No. 3 and vacuole formation in No. 4 only.

Group II. Nerve Section and Digitalis.—The animals were operated on in the manner described and the pulse increase determined by the electrocardiogram. Ten days postoperative digifoline (Ciba, 2 c.c. equal 1 cat unit) injections were begun and each animal received two minims per pound of body weight daily. This was gradually increased until they received four to six minims per pound of body weight. Rabbit No. 14 had a pulse increase of 20 with the digitalis and died of over digitalization (Table II).

At the end of the sixty-day observation period, the three surviving animals were killed. Gross examination of the hearts revealed that the muscle was moderately firm, except in No. 14. Epicardial fat was scant or absent. The right heart chambers were dilated and the hearts appeared elongated. The heart weight averaged 7.7 grams. The ventricular length was 2.7 cm., the thickness of the left ventricle 6.5 mm. above and 5 mm. below, with a diameter of 1.6 cm.

TABLE II

GROUP II. EXPERIMENTAL DATA CONCERNING RABBITS WITH CARDIAC INHIBITION REMOVED AND DIGITALIS INJECTED

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS	PULSE CHANGE	DEATH	HEART WEIGHT	LEFT VENTRICLE LENGTH	LEFT VENTRICLE ABOVE	LEFT VENTRICLE BELOW	LEFT VENTRICLE RING	LEFT VENTRICLE DIAM.	LEFT VENTRICLE CIRC.	RIGHT VENTRICLE LENGTH	RIGHT VENTRICLE ABOVE	RIGHT VENTRICLE BELOW	RIGHT VENTRICLE DIAM.	RIGHT VENTRICLE CIRC.	ECG	COURSE
10	2.6	60	250	0	120	K	6	19	7	5	10	12	23	19	1	0.5	12	32	P.O. Rate 270 A.D. Rate 150 Slurred QRS	Abscess at site of injection
12	2.6	60	200	0	180	K	8	27	6	5	11	13	26	27	1	0.7	13	34	P.O. Rate 280 A.D. Rate 160 Slurred QRS	Negative
15	2.5	60	170	0	160	K	7.8	25	8	5	13	20	35	25	2	1	20	30	P.O. Rate 230 A.D. Rate 150 Isoelectric T waves	Negative
14	2.86	28	190	0	130	D	9	24	6	4	13	20	32	24	2	1	20	33	P.O. Rate 210 A.D. Rate 220 Depressed S-T intervals	Death due to overdigitalization

P.O. is the postoperative rate.
A.D. is the rate after digitalis.

The general findings on microscopic examination included poor staining of the nuclei, moderate separation of the muscle bundles with hyalinization, marked granular degeneration and fibrosis. In No. 10, where the animal developed a localized abscess in the left thigh, the findings were especially marked with vacuole formation, granular degeneration and separation of the muscle fibers.

Group III. Nerve Section and Quinidine.—The animals were operated in the manner described. Fourteen days postoperative feedings of quinidine sulfate were begun. The first doses were gr. ss, which was increased to gr. i after two weeks' medication (Table III). In the four rabbits autopsied at the end of the observation period, gross examination of the hearts revealed a firm muscle, purple-gray in color, and scant epicardial fat. The average heart weight was 5.7 gm. The length was 2 cm.; with thickness of the left ventricle 4.5 mm. above and 3.5 mm. below, and a diameter of 1.6 cm. Microscopic examination revealed essentially a moderate degree of granular degeneration and vacuolization, with a slight degree of fibrosis and hyalinization, separation of the muscle bundles, and fragmentation of a moderate degree.

Group IV. Thyroid Feedings.—Toxicosis was produced in these animals by feedings of desiccated thyroid. All of the animals showed emaciation and cachexia. On gross examination (Table IV) the heart muscles were moderately firm and devoid of epicardial fat. The right heart chambers were dilated and the hearts appeared elongated. Grayish areas were prominent over the external surfaces of the left ventricles. The average weight was 4.3 grams, length 2.1 cm., thickness of the left ventricle 4.8 mm. above and 3.3 mm. below, with a diameter of 1.3 cm.

In the animals that died early due to toxicosis there were no histologic changes prominent on the microscopic examination. There were focal areas of early degeneration, hyalinization, and slight separation of the muscle fibers. In the other animals the changes were more marked. These showed areas of marked granular and fatty degeneration with a foamy appearance, marked separation of the muscle fibers with fraying and fragmentation, cullular infiltration, both round cell and neutrophilic, hyalinization and fibrosis of these areas.

Group V. Thyroid and Digitalis.—These rabbits were given the digifoline subcutaneously for four days prior to the time the desiccated thyroid feedings were begun. The feedings were continued until the symptoms and signs of toxicosis became evident; the digitalis was continued each day also. None survived the toxicosis; all showed evidence of severe emaciation (Table V). Gross examination revealed elongated hearts with the muscles purple-gray in color and moderately firm. The right chambers were dilated and filled with clots. Epicardial fat was very scant or absent. The average heart weight was 5 grams. The length of the left ventricle was 2.2 cm.; thickness, 4.2 mm. above and 3.3 mm. below, with a diameter of 1.42 cm.

Where the animals died within twelve to sixteen days, following eight to twelve days of thyroid feedings, the histologic changes in the heart muscle were not marked on microscopic examination. There were focal areas of hyalinization and moderate granular degeneration with fragmentation and

TABLE III

GROUP III. EXPERIMENTAL DATA CONCERNING RABBITS WITH CARDIAC INHIBITION REMOVED AND QUINIDINE ORALLY

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS	PULSE CHANGE	DEATH	HEART WEIGHT	LEFT VENTRICLE LENGTH	LEFT VENTRICLE ABOVE	LEFT VENTRICLE BELOW	LEFT VENTRICLE RING	LEFT VENTRICLE DIAM.	RIGHT VENTRICLE CIRC.	RIGHT VENTRICLE LENGTH	RIGHT VENTRICLE ABOVE	RIGHT VENTRICLE BELOW	RIGHT VENTRICLE DIAM.	QUINIDINE GIVEN	ECG	COURSE
19	2.6	54	170	.15 kg.	+70	K	6.7	22	5	4	12	15	31	22	2	1	15	38 grains	Increased amplitude of T waves	Negative
21	2.6	51	160	.43 kg.	+80	K	7.0	20	5	4	13	19	33	20	1.5	1	19	38 grains	Increased amplitude of T waves	Negative
31	1.5	51	210	0	+30	K	4.8	20	4	3	12	16	30	20	2	1	16	88 grains	Increased amplitude of T waves	Negative
32	1.6	47	220	.4 kg.	170	D	5.2	19	4	3	11	14	24	19	1	.5	14	72 grains	Increased amplitude of T waves	Negative

TABLE IV

GROUP IV. EXPERIMENTAL DATA CONCERNING RABBITS GIVEN DESICCATED THYROID

ANIMAL NO.	WEIGHT KG.	DAYS	PULSE	WEIGHT LOSS KG.	PULSE CHANGE	DEATH	HEART WEIGHT	LEFT VENTRICLE LENGTH	ABOVE	BELOW	RING	DIAM.	RIGHT VENTRICLE CIRC.	LENGTH	ABOVE	BELOW	DIAM.	NO. OF TOXICOSIS	THYROID GIVEN	E.C.G.	COURSE
25	1.5	60	170	.5	+70	D	4.6	21	5	4	10	11	22	21	2	1.5	11	2	22 grains	High T's	Toxicosis
26	1.2	3	160	.2	?	D	3.2	20	4	3	9	13	23	20	1	1.0	13	1	5 grains	High T's	Toxicosis
27	1.3	5	150	.37	?	D	3.2	19	5	3	9	12	21	19	1	1.0	12	1	6 grains	High T's	Toxicosis
36	1.4	12	210	.4	0	D	4.1	14	5	3	9	12	24	14	2	1.0	12	1	75 grains	Normal	Toxicosis
37	1.3	14	200	.4	+40	D	3.7	20	5	3	10	11	21	20	1	1.0	11	1	93 grains	High T's	Toxicosis
38	1.4	50	250	.5	+110	D	5.3	23	5	3	12	15	28	23	1.5	1.0	12	2	118 grains	and QRS High T's	Toxicosis Toxicosis
39	1.7	56	210	.3	+180	K	5.8	23	4	3	13	14	32	23	1.5	1.0	14	2	138 grains	and QRS High T's	Toxicosis Toxicosis
41	1.2	13	210	.4	+20	D	3.8	18	4	3	11	13	23	18	2	1.0	13	1	87 grains	High T's	Toxicosis
40	1.3	42	240	.35	+90	D	4.4	20	5	1	10	12	20	20	2	1.5	10	2	274 grains	High T's	Toxicosis
35	1.6	49	250	.4	+100	D	5.0	23	6	4	12	15	32	23	2	1.0	15	2	301 grains	High T's	Toxicosis

TABLE V

GROUP V. EXPERIMENTAL DATA CONCERNING RABBITS FED DESICCATED AND DIGITALIS INJECTED

								Left Ventricle						Right Ventricle							
Animal No.	Weight Kg.	Days	Pulse	Pulse with Digitalis	Pulse with Thyroid	Death	Heart Weight	Length	Above	Below	Ring	Diam.	Circ.	Length	Above	Below	Diam.	Digitalis C.C.	Thyroid Grains	ECG	Course
46	1.7	18	220	220	310	D	4.8	22	5	4	12	14	29	22	2	1	14	36	126	T waves + to −	Toxicosis
47	1.2	12	200	240	320	D	4.3	21	4	3	11	13	26	21	2	1	13	28	83	T waves negative	Toxicosis
48	1.1	16	210	210	280	D	4.1	20	3	2	10	15	29	20	1.5	1	15	32	72	P-R prolonged	Toxicosis
49	1.3	16	270	230	340	D	6.0	21	4	3	12	16	30	21	2	1	16	32	72	T isoelectric	Toxicosis
50	1.6	15	220	240	310	D	5.6	20	5	4	11	13	26	20	2	1	13	30	147	Low S-T	Toxicosis

TABLE VI

GROUP VI. EXPERIMENTAL DATA CONCERNING RABBITS FED DESICCATED THYROID AND QUINIDINE

								Left Ventricle						Right Ventricle							
Animal No.	Weight Kg.	Days	Pulse	Pulse with Quinidine	Pulse with Thyroid	Death	Heart Weight	Length	Above	Below	Ring	Diam.	Circ.	Length	Above	Below	Diam.	Quinidine Grains	Thyroid Grains	ECG	Course
51	1.4	10	220	210	310	D	4.7	23	3	2	11	13	25	23	1.5	1	13	20	58	No change	Toxicosis
52	1.7	15	200	200	270	D	5.5	22	4	2	12	13	25	22	2.0	1	13	30	105	No change	Toxicosis
53	1.2	10	170	140	250	D	6.3	22	3	2	13	17	28	22	1.5	1	17	20	55	No change	Toxicosis
54	1.7	10	180	210	290	D	6.5	22	4	3	10	15	26	22	1.0	.75	15	20	70	High T's	Toxicosis
55	1.6	20	180	170	320	D	6.1	22	6	4	10	15	25	22	1.5	1	15	40	170	High Voltage	Toxicosis

fraying of the muscle. Round and neutrophilic cellular infiltration were noted in two of the animals. The rabbit, No. 46, that lived the longest, 18 days, showed the most marked degenerative changes, which were fatty and graular, with vacuolization, moderate fibrosis, and marked neutrophilic and endothelial cellular exudation.

Group VI. Thyroid and Quinidine.—These animals were first fed quinidine for four days before desiccated thyroid was given. Both were then fed until the symptoms and signs of toxicosis were manifested. All of the animals died during the first toxicosis (Table VI). Gross examination revealed elongated hearts with dilated right chambers and scant epicardial fat. The muscles were purple-red and firm. The average heart weight was 5.8 grams. Average length was 2.2 cm.; thickness, 5 mm. above and 2.1 mm. below; and the average ventricular diameter was 1.5 cm. Many marked histologic changes were noted on microscopic examination. These consisted of moderate to marked fraying and fragmentation of the muscles with hyaline and granular degeneration, and cellular infiltration of the round and neutrophilic types.

ANALYSIS OF EXPERIMENTAL DATA

Several interesting points were brought out in this experimental study. In Group I, where the operation for the removal of cardiac inhibitory nerves was performed on 11 rabbits, only four survived the observation period of sixty days. Here there was only the increased heart rate with increased work for the myocardium. Apparently the tachycardia alone caused an increase in the ventricular measurements of the animals that lived sixty days. In Group II, animals that had the nerve section performed and received digitalis ten days later, the picture was practically similar. The average heart weight was not as high as in the first group, the cardiac rate having decreased after the tenth postoperative day due to the digitalis. In Group III, where the nerves were removed and quinidine fed, no great deviation was noted in the anatomical findings.

Cardiac changes became more manifest in the last three groups that were fed desiccated thyroid. The average weight loss was about the same. There was no doubt as to the evidence of an increased metabolism accompanied by an increased respiratory rate and an accelerated heart action. The longer the animals lived with the toxicosis, the greater were the cardiac changes. The hearts appeared elongated, and the histologic changes were considerably greater than in those animals who received no thyroid feedings.

COMMENT

There are many factors to be considered in the effects of thyrotoxicosis on the heart. It is generally agreed that thyrotoxicosis produces general muscular weakness. It uses up glycogen reserves so that endurance is markedly lessened from the overactivity. The heart usually shares this decrease in glycogen reserve and suffers according to the extent and duration of the loss. Tachycardia is due mainly to the increase of thyroxin in the myocardium which causes the heart to beat more rapidly and more vigorously.[6] However, tachycardia alone is not sufficient to cause marked cardiac alteration. It may produce

some changes, such as fragmentation and degeneration of the muscle, depending on its duration. It is a factor, secondary to thyroxin.

There was a great variety and a marked degree of alterations in the heart muscle. Whether the compensating and neutralizing capacity of the neuro-endocrine system accounts for some of the variations can only be theorized at this time. The quantitative results were too variable for a direct ratio correlation. Even in the experimental animal, there is a different degree of tolerance to thyroid medication, and it is plausible that this is caused by a synergistic and antagonistic interrelation of other endocrine glands.

Experimentally, in rabbits, thyroid extract produces definite myocardial changes in varying amounts. These changes are not proportional to the alteration of the heart rate alone nor dependent on a previous demonstrable infection. Nor is the myocardial damage proportional to the increased rate of speed per second.[5] Even though the cardiac lesion in thyrotoxicosis may not be specific or constant, the myocardial damage encountered, however the mechanism, is primarily due to the effects of increased thyroid on the heart muscle itself.

SUMMARY

Experimentally induced thyrotoxicosis in rabbits resulted in the following changes in the heart: parenchymatous and fatty degeneration, cellular invasion, fraying and fragmentation of the heart muscle, and fibrosis. Similar changes, but not as marked, were produced by cardiac overwork without an excess of thyroxin in the circulation. This was produced by cutting the depressor nerves and denuding the carotid sinuses of their investments in order to allow the heart to operate uncontrolled.

A heart that is induced to work more rapidly does respond with morbid anatomical changes, but these cardiac alterations are not as marked as those due to the summation of the effect of increased thyroxin on the myocardium and the tachycardia, which is again a thyroxin effect.

REFERENCES

1. (a) Baust, H.: Ueber die histologische Befund in Kropfherz, Beitr. z. path. Anat. u. z. allg. Path. 86: 543, 1930.
   (b) Chesky, V. E.: Cardiac Manifestations of Goiter, Southwestern Med. 13: 79, 1929.
   (c) Rautmann, H.: Pathologisch-anatomische Untersuchungen über die Basedowsche Krankheit, Mitt. a. d. Grenzgeb. d. Med. u. Chir. 28: 487, 1914.
   (d) Weller, C. V., Wanstrom, R. C., Gordon, H., and Bugher, J. C.: Cardiac Histopathology in Thyroid Disease; Preliminary Report, Am. Heart J. 8: 8, 1932.
2. (a) Askanazy, M.: Pathol. anat. Beiträge zur Kenntnis des Morbus Basedowii, imbesondere über die dabei auftretende Muskelerkrankung, Deutsche Arch. f. klin. Med. 61: 118, 1898.
   (b) Ceelen, W.: Ueber Herzvergrösserungen in frühen Kindersalter, Berl. klin. Wchnschr. 57: 213, 1920.
   (c) Fahr, T.: Hist. Befunde am Kropfherzen, Zentralbl. f. allg. Path. u. path. Anat. 27: 1, 1916.
   (d) Goodpasture, E. W.: Myocardial Necrosis in Hyperthyroidism, J. A. M. A. 76: 1545, 1921.
   (e) Loos, F.: Kasuitischer Beitrag zur Frage der Herzveränderungen bei Morbus Basedowii, Ztschr. f. Kreislaufforsch. 21: 641, 1929.
   (f) Wilson, L. D.: Note on the Pathology of Hearts, etc., Tr. A. Am. Physiol. 38: 144, 1923.
3. (a) Lerman, J., and Means, J. H.: Cardiovascular Symptomatology in Exophthalmic Goiter, Am. Heart J. 8: 55, 1932.
   (b) Maher, C. C., and Sittler, W. W.: The Cardiovascular State in Thyrotoxicosis, J. A. M. A. 106: 1546, 1936.
   (c) McDonald, C. H.: The Work of the Hyperthyroid Heart, Am. Heart J. 13: 687, 1937.

4. (a) Cameron, A. T., and Carmichael, J.: Comparative Effects of Para-Thyroid and Thyroid Feedings on Growth and Organ Hypertrophy in White Rats, Am. J. Physiol. 58: 1, 1921.
(b) Goodpasture, E. W.: The Influence of Thyroid Products on the Production of Myocardial Necrosis, J. Exper. Med. 34: 407, 1921.
(c) Hashimoto, H.: The Heart in Experimental Hyperthyroidism, With Special Reference to Its Histology, Endocrinology 5: 579, 1921.
(d) Takane, K.: Ueber die experimentelle akute Myocarditis durch Thyroidin und Jodsalze, Virchows Arch. 259: 737, 1925.
5. Menne, F. R., Keane, R. H., Henry, R. T., and Jones, N. W.: The Heart in Hyperthyroidism—An Experimental Study, Am. Heart J. 8: 75, 1932.
6. Yater, W. M.: The Mechanism of Adjustment of the Circulation in Hyperthyroidism (Thyrotoxicosis), Am. Heart J. 8: 1, 1932.

FATAL ANURIA FOLLOWING ADMINISTRATION OF SULFONAMIDES WITH REFERENCE TO TUBULAR NECROSIS AND REGENERATION*

JANE A. ERGANIAN, M.D., AND JOHN H. DOVAL, M.D.
ST. LOUIS, MO.

IN MOST reports on the effects of the sulfonamide drugs on the urinary tract, emphasis has been given to the deposition of crystals in the tubules of the kidney and the formation of concrements in the pelves and ureters. Less emphasis has been given to the direct toxic effect of these drugs on the renal epithelium. In experiments on the toxicity of sulfathiazole in mice, Rake, van Dyke, and Corwin[1] found only an occasional mouse in which there were advanced degenerative changes and necrosis in the epithelium of the proximal convoluted tubules. Similar observations are reported by Kolmer[2] in studies with sulfathiazole in rabbits. Tubular necrosis was found only in animals receiving the largest doses—0.2 Gm. per kilogram twice a day for ten days. Hellwig and Reed[3] emphasize the toxic effect of sulfadiazine on the convoluted tubules and distinguish between this effect and mechanical blocking of the tubules by crystals. The latter effect is stressed by Bradford and Shaffer,[4] who accent the need for early ureteral catheterization and pelvic lavage. Hellwig and Reed insist that lavage in their case was of no avail—that the lesion was one of toxicity instead of mechanical effect.

In a recent case studied at the St. Louis Children's Hospital the preponderance of clinical and pathologic evidence indicates that death from renal insufficiency was directly related to degenerative changes in the renal epithelium, and that the deposition of crystals in the kidney and pelvis was of minor importance.

CASE REPORT

G. S., a boy of nine months, was seen first in the Out-Patient Department on March 10, 1942, with acute rhinopharyngitis, bilateral catarrhal otitis media, and bronchitis. His temperature was 39° C. Sulfathiazole 0.25 Gm. every four hours (0.2 Gm. per kilogram), neo-

*From the Departments of Pathology and Pediatrics, Washington University School of Medicine, and the St. Louis Children's Hospital.

Received for publication, August 17, 1942.

synephrin nose drops, and an adequate fluid intake were prescribed. The temperature returned to normal within twenty-four hours, the infection cleared rapidly, and the sulfathiazole was discontinued on the fifth day. On this same day gross hematuria and sulfathiazole crystals in the urine were noticed, both of which disappeared within the next twenty-four hours. Three days later the temperature rose again to 39° C. because of recurrence of the otitis media. This time treatment was by bilateral myringotomy and sulfadiazine, 0.25 Gm. every four hours. The temperature again returned to normal within twenty-four hours, and the otitis media began to subside. No hematuria was noticed. On the fourth day of the relapse, vomiting occurred, and when the patient was hospitalized, examination revealed marked oliguria, considerable albuminuria, and 4 to 5 red blood cells per high-power field in a centrifuged specimen but no crystals of sulfadiazine. During the second twenty-four hours of hospitalization the temperature rose, only a few cubic centimeters of urine were voided, and generalized edema developed. The heart sounds were normal. Late the second day the kidneys were found to be enlarged and tender. On the third day the heart tones became poor, cyanosis and marked restlessness developed, and the child died fifty-four hours after hospital entry, despite administration of magnesium sulfate, oxygen, and sedation. Additional laboratory work included a negative Kline test; hemoglobin 11.3 Gm.; red blood cell count 4.5 million; white blood cell count 13,000; and Schilling differential of 6 per cent stabs, 55 per cent segmented, 37 per cent lymphocytes, and 2 per cent monocytes.

TABLE I

DAY	CHEMOTHERAPY	URINE	DRUG LEVEL (MG./100 C.C.) BLOOD	URINE
March 10	Sulfathiazole 0.25 Gm./4 hr.			
March 11, 12, 13	Same			
March 14	Same	Gross hematuria Numerous crystals		
March 15	None	Cleared gradually		
March 16	None	Clear*		
March 17	None	Clear*		
March 18	Sulfadiazine 0.25 Gm./4 hr.	Clear*		
March 19, 20	Same	Clear* Amount (?)*		
PATIENT HOSPITALIZED				
March 21	Same	Oliguria noted	Free 17.7 Total 21.2	
March 22	None	Marked oliguria Albumin, +2 R.B.C. centrifuged Crystals—none		
March 23	None	Marked oliguria	A.M.— Free 15.8 Total 29.4 P.M.— Free 16.5 Total 20.5	Free 168.0 Total 375.0

*As observed by the mother.

The autopsy (Washington University No. 9746) was performed two and one-half hours post mortem. The body was edematous; the face, especially about the eyes, was puffy due to subcutaneous edema. The serous cavities contained excessive amounts of clear yellow fluid without evidence of inflammatory lesions within them. The kidneys were similar; the right weighed 55 Gm. and the left 53 Gm. The capsules were tense, and during the process of section of the organs large amounts of reddish yellow fluid spurted around the cutting edge

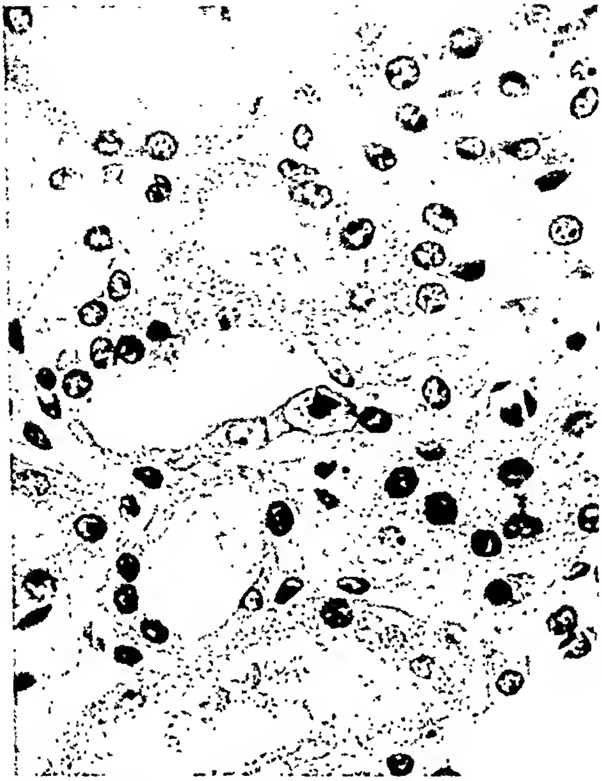


Fig. 1.—A mitotic figure is present in an epithelial cell in the center. In other tubules the cell membranes are indistinct and nuclei have disappeared.

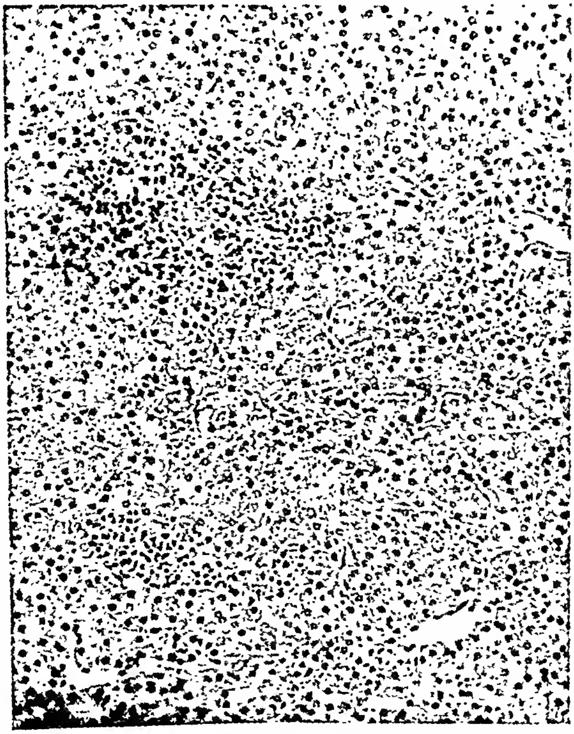


Fig. 2.—Focal necrosis in the liver. The hepatic cells are destroyed in these areas and there is an infiltration of leucocytes, lymphocytes, and a few monocytes.

of the knife. Approximately 10 c.c. of fluid were in the pelves and calices of these kidneys. The architecture of the organs was not unusual. There was a slight amount of dilatation of the calices and pelves. In the grayish white mucosa of the pelves were petechiae, and on the surface of the mucosa were many small yellow crystals. At the orifices of the ureteropelvic junctions were large aggregations of these crystals. These crystals were also found on the ureteral mucosa. The ureters were otherwise normal. The urinary bladder contained no urine. The remainer of the gross pathologic findings are listed below in the complete pathologic diagnosis.

Microscopic examination of the kidneys reveals moderate necrosis of the epithelium of the tubules, particularly of the proximal convoluted tubules. Within the lumen of the tubules are desquamated cells, eosinophilic debris, and occasionally red blood cells. Often there are syncytial masses of cytoplasm congregated at one end of a section of tubule with 4 to 8 well-preserved chromatic nuclei. Mitotic figures in the epithelial cells of the proximal tubules are moderately abundant (Fig. 1). Near the pelvic margins of the medullary papillae is noticeable dilatation of the tubules. Hemorrhage is present beneath the mucosa and within the adjacent tubules. The glomeruli are of moderate size, and the epithelial cells of the visceral layer of Bowman's capsule are cuboidal in shape, with deeply chromatic nuclei. Within Bowman's space there are a few desquamated necrotic cells and eosinophilic debris. There is slight interstitial edema of the cortex.

In the liver are many areas of focal necrosis (Fig. 2). These areas are small and contain necrotic liver cells, leucocytes, lymphocytes, and a few mononuclear cells. There is a moderate amount of cloudy swelling and fatty degeneration of the hepatic cells.

The complete pathologic diagnosis is as follows: Primary: Acute nephrosis with early regeneration of tubular epithelium; crystalline material in the pelves and ureters; hydronephrosis, bilateral; petechiae and ecchymoses of the mucosa of the pelves of the kidneys; edema of the subcutaneous tissues; ascites; hemorrhage of the middle lobe of the right lung; petechiae of the myocardium; hydrothorax, bilateral; focal atelectasis of the lungs; hyperplasia of the intrapulmonary lymphoid follicles; partially organized thrombi in the smaller branches of the pulmonary arteries. Accessory: Patent foramen ovale, guarded type, and immaturity of the kidneys.

COMMENT

The pathologic findings in the kidneys of this child indicate that there are two distinct lesions: one the result of crystallization of sulfathiazole in the ureters, pelves, and tubules; and the other, necrosis of the epithelium of the proximal convoluted tubules with early regeneration. It is not possible to attribute the anuria to either of these exclusively, but the preponderance of evidence indicates that the degenerative changes in the renal epithelium are more important. This conclusion is supported by the finding of regeneration, which might be expected to occur on the eighth to the tenth day following primary damage to the kidney, as in the case of poisoning with bichloride of mercury. In this patient the first sulfonamide was administered on March 10, and four days later there were gross hematuria and numerous crystals in the urine. Oliguria appeared on March 21, eleven days after the administration of sulfathiazole and three days after the first dose of sulfadiazine. It is improbable that the renal epithelium could have been damaged and could have showed beginning repair within the period of five days. It must then be concluded that the injury to the kidney resulted from the sulfathiazole given from March 10 to March 14, inclusive.

The sulfathiazole was given in therapeutic amount, and the reason for the damage to the kidney is not evident. It is possible that the mother did not give

the child an adequate amount of water, and that the concentration of the acetylated derivative in the urine was greater than in the usual case. There is also the possibility that the relative immaturity of the kidney may have played a role. The epithelium covering the visceral layer of Bowman's capsule was, in this nine-month-old child, still of the cuboidal type seen in newborn infants.

SUMMARY

1. A case of fatal anuria following the administration of therapeutic doses of sulfathiazole is reported.

2. An analysis of the pathologic findings indicates that injury to, and necrosis of, the renal epithelium were of greater physiologic importance than the usually emphasized formation of concrements and obstruction of the ureter and tubules.

REFERENCES

1. Rake, G., van Dyke, H. B., and Corwin, W. C.: Pathologic Changes Following Prolonged Administration of Sulfathiazole and Sulfapyridine, Am. J. M. Sc. **200**: 353, 1940.
2. Kolmer, J. A.: The Pathologic Tissue Changes Produced by Sulfathiazole and Sulfathiazoline in Rabbits, J. Lab. & Clin. Med. **27**: 1012, 1942.
3. Hellwig, C. A., and Reed, H. L.: Fatal Anuria Following Sulfadiazine Therapy, J. A. M. A. **119**: 561, 1942.
4. Bradford, H. A., and Shaffer, J. H.: Renal Changes in a Case of Sulfadiazine Anuria, J. A. M. A. **119**: 316, 1942.

CIRCULATION TIME: A REVIEW OF PREVIOUS METHODS AND THE INTRODUCTION OF AMINOPHYLLIN AS A NEW AGENT

Harry Koster, M.D., F.A.C.S., and Stanley J. Sarnoff, M.D.
Brooklyn, N. Y.

TECHNICAL difficulties have thus far interfered with direct measurements of the velocity of blood in the human being. An approximation has been suggested by the estimation of the "circulation time" which obviously varies inversely with the velocity. The estimations of circulation time are dependent upon the detection of the arrival at some previously chosen point of a substance introduced elsewhere.

The arrival of the substance may be detected by the patient, by the observer, or both. In general, it is expected that objectivity represents not only greater accuracy, but also wider applicability (children, uncooperative or comatose patients).

The earliest effort to measure circulation time was that of Hering[1] in 1827. Using the Prussian blue method he measured the time lapse between the injection of potassium ferrocyanide in one jugular and its appearance in the other.

From Crown Heights Hospital, Brooklyn.
Received for publication, Aug. 28, 1942.

In 1858 Vierordt[2] amplified and extended the use of this method and, after many experiments in different animals, predicted that in a human being with a heart rate of 72 per minute the circulation time from jugular to jugular would be 23.1 seconds.

In 1893 Stewart[3] injected methylene blue intravenously and detected its arrival elsewhere by transillumination. He also injected hypertonic sodium chloride solution, the presence of which was determined by the use of a galvanometer connected to nonpolarizable electrodes placed on a vessel. Meldolesi,[4] in 1925, and Koch,[5] in 1928, used the same method.

In 1922 Loevenhart[6] and his co-workers injected sodium cyanide intravenously and used a respiratory gasp as the end point at which time the cyanide was considered to have reached the carotid sinus. In 1933 Robb and Weiss[7] adapted this method for use in human beings. In normal subjects they found it to vary between 9 and 21 seconds, with an average of 15.6. The foot-to-carotid time averaged 27.7 seconds. This circulation time includes the time taken for the reaction to occur after the arrival of the cyanide at the carotid sinus. Olson, Levinson, Necheles, and Gutmann[8] in a study of circulation time in shock showed that the "reactivity time" of sodium cyanide varied constantly between 1 and 2 seconds.

In 1927 Blumgart and Yens[9, 10] devised a method for determining vein-to-artery and vein-to-heart time which made it possible to calculate the circulation time in the pulmonary circuit. They injected radium C into an antecubital vein and by means of a modified Wilson cloud chamber observed the time required to reach the heart and the opposite brachial artery, near the elbow. Blumgart and Weiss[11] found that the time from vein to artery varied from 12 to 24 seconds, with an average of 18 seconds. The pulmonary circuit time varied from 5 to 17 seconds, with an average of 10.8 seconds. This method undoubtedly is the most accurate one available, but its complexity makes its clinical use unfeasible.

Weiss, Robb, and Blumgart,[12] in 1929, found that the antecubital injection of histamine phosphate produced a flushing of the face upon the arrival of the histamine in the minute vessels. The arm-to-face time averaged 24 seconds. The method has been abandoned because of dyspnea, cardiac asthma, and headache as reaction phenomena.

Winternitz, Deutsch, and Brüll[13, 14] found that the injection of a solution of sodium dehydrocholate (decholin) produced a bitter taste upon arriving in the tongue capillaries. Tarr, Oppenheimer, and Sager[15] found the circulation time by this method to vary between 10 and 16 seconds.

In 1933 Fishberg, Hitzig, and King[16] used saccharin to measure the arm-to-tongue circulation time. A sweet taste signals the end point. The time by this method varies between 9 and 16 seconds.

In 1935 Teplov and Sor[17] used lobeline with a cough as the end point. They had from 30 to 40 per cent failures. The method was improved by Piccione and Boyd[18] who used a tickling sensation in the throat as an end point.

In 1935 Hitzig[19] used the injection of ether to measure the arm-to-lung circulation time. The detection of the ether in the expired air by the patient and often by the observer signals the end point. The lapse of time varies between 4 and 8 seconds.

In 1936 Goldberg[20] injected a solution of calcium gluconate and found that by using the sensation of heat on the tongue as an end point the arm-to-tougne circulation time averaged 12.4 seconds, varying between 10 and 16 seconds.

In 1936 Spier, Wright, and Saylor[21] injected a solution containing magnesium sulfate, calcium gluconate, sodium chloride, and copper sulfate. The arrival of the solution at various stations was signaled by a sensation of heat. They found the average circulation time of the arm to the tongue to be 14.6 seconds, to the hands 26 seconds, and to the feet 28 seconds.

In 1935 Zwillinger[22] suggested the use of magnesium sulfate as the test drug. In 1937 Neurath[23] employed this drug extensively, using the sensation of warmth in the head as the end point.

In 1939 Gubner, Schnur, and Crawford[24] used the inhalation of carbon dioxide to measure the circulation time from the pulmonary capillaries to the head. The end point was a feeling of warmth in the head and an acceleration and increased depth of respiration. This time ranged from 5 to 10 seconds.

In 1922 Koch[25] injected fluorescein to measure arm-to-arm time and found it to vary from 12 to 26 seconds.

Recently Fishbach[26] reported the arm-to-eye time estimated by the injection of sodium fluorescein to vary between 7 and 15.6 seconds. The end point is the appearance of a bright yellow fluorescence in the lower conjunctiva when viewed by ultraviolet light in a dark room.

A critical review of these methods strongly suggests the desirability of establishing criteria for the acceptance of any method. The most desirable method must conform to the following requirements:

1. The injected material must be nontoxic in the doses used.

2. The injection of a small quantity (1 c.c. or less) should be sufficient to give a sharp end point so that inaccuracies resulting from time loss during the injection be avoided.

3. The end point should be sharp and reliable.

4. The end point should be objective to eliminate the source of error inherent in the untrained observer and the possible sluggishness of mental reaction of the patient and to extend the applicability of the method to children, the uncooperative, and the comatose.

5. The minimum of apparatus in simple form is necessary to facilitate clinical use.

6. The agent used should be readily available.

The xanthines have long been known to be respiratory stimulants. The observation that the intravenous injection of one of them, aminophyllin, produced a sharp respiratory gasp suggested its use for the determination of arm-to-head circulation time.

METHOD

The patient should be lying flat on the back for the brief duration of the test. A No. 20 gauge needle is introduced into a vein in the antecubital fossa. The tourniquet is released and thirty seconds later 1 c.c. of aminophyllin (0.24 Gm.) is rapidly injected while a stop watch marks the moment of beginning of the injection. The awaited end point is a marked increase in the depth of inspiration which in many cases amounts to a gasp. While this occurs in every instance, in some it may be preceded by swallowing movements, a change in facial expression resulting from subjective sensations, or sharp catch during expiration. The first observable change is taken as the end point.

RESULTS AND DISCUSSION

Ninety-two patients at various stages in the postoperative state were subjected to this test. This included males and females ranging in age from 12 to 82 years.

The characteristic reaction of the patient was a transient dizziness, flushing, and a hyperpnea. In no case did this fail to pass off in a few minutes, and no other untoward effects were observed.

The circulation times varied from 7.1 to 20.4 seconds, averaging 12.4 seconds.

This test under the limited conditions of trial here described has been perfectly safe. No special apparatus is required.

The preparation is available in 2 c.c. ampules previously recommended for intramuscular use. Although as little as 0.25 c.c. is frequently sufficient to give an end point as determined by preliminary studies, the larger dose of 1 c.c. is preferable because it always gives the response, and yet it is still a small enough quantity to be quickly injectible.

Confirmation of the respiratory response to the intravenous injection of aminophyllin is found in the report of Sperling, Weisman, and Papermaster[27] who used it postoperatively in the hope of diminishing atelectasis and pneumonia. They graphically demonstrated the response by recording the respiratory excursion with the ordinary basal metabolism machine. In a study of twenty-two patients they found "an immediate increase of the depth of respiration averaging 51 per cent."

It is an objective method with a sharp end point. In the seventy-two instances in which it was used by us, in only one case was there any difficulty in recognizing the end point.

REFERENCES

1. Hering: Ztsch. J. Physiol. 3: 85, 1827.
2. Vierordt: Der Erscheinungen und Gesefze der stromgeschwindig Keiter des Blutes, Frankfort A. M., 1858.
3. Stewart: Researches on the Circulation Time in Organs and the Influences Which Affect It, J. Physiol. 15: 31, 1893.
4. Meldolesi: Bull. e. ath della reale academica med. de Rome 52: 267, 1925-1926.
5. Koch: Handb. d. biol. Arbeits methoden, 1928, p. 345.
6. Loevenhart, A. S., Schlomovitz, B. H., and Seybold, E. G.: Determinations of Circulation Time in Rabbits and Dogs and Its Relation to Reaction Time of Respiration to Sodium Cyanide, J. Pharmacol. & Exper. Therap. 19: 221, 1922.

7. Robb, G. P., and Weiss, S.: Method for Measurement of Velocity of Pulmonary and Peripheral Venous Blood Flow in Man, Am. Heart J. 8: 650, 1933.
8. Olson, W. H., Levinson, S. O., Necheles, H., and Gutmann, H.: Circulating Time in Shock, War Med. 1: 830, 1941.
9. Blumgart, H. L.: Velocity of Blood Flow in Health and Disease, Velocity of Blood Flow in Man and Its Relation to Other Measurements of Circulation, Medicine 10: 1, 1931.
10. Blumgart, H. L., and Yens, O. C.: Studies on Velocity of Blood Flow; Method Utilized, J. Clin. Investigation 4: 1, 1927.
11. Blumgart, H. L., and Weiss, S.: Studies on Velocity of Blood Flow, Velocity of Blood Flow in Normal Resting Individuals and Critique of Method Used, J. Clin. Investigation 4: 15, 1927.
12. Weiss, S., Robb, G. P., and Blumgart, H. L.: Velocity of Blood Flow in Health and Disease as Measured by Effect of Histamine on Minute Vessels, Am. Heart J. 4: 664, 1929.
13. Winternitz, M., Deutsch, J., und Brüll, Z.: Eine klinisch brauchbare Bestimmungsmethode der Blutumlaufszeit mittels Decholeninjektion, Erwiderung 27: 986, 1931.
14. Winternitz, M., Deutsch, J., und Brüll, Z.: Eine klinisch brauchbare Bestimmungsmethode der Blutumlaufszeit mittels Decholeninjektion. Erwiderung, Med. Klin. 28: 831, 1932.
15. Tarr, L., Oppenheimer, B. S., and Sager, R. V.: Circulation Time in Various Clinical Conditions Determined by the Use of Dehydrocholate, Am. Heart J. 8: 766, 1933.
16. Fishberg, A. M., Hitzig, W. M., and King, F. H.: Measurement of Circulation Time With Saccharin, Proc. Soc. Exper. Biol. & Med. 30: 651, 1933.
17. Teplov and Sor: Teraperticeski Arch. 13: 57, 1935.
18. Piccione, F. V., and Boyd, L. J.: Determination of Blood Velocity by Lobeline, J. Lab. & Clin. Med. 26: 766, 1941.
19. Hitzig, W. M.: Use of Ether in Measuring Circulation Time From Antecubital Veins to Pulmonary Capillaries, Am. Heart J. 10: 1080, 1935.
20. Goldberg, J. S.: Use of Calcium Gluconate as Circulation Time Test, Am. J. M. Sc. 192: 36, 1936.
21. Spier, L. C., Wright, I. S., and Saylor, L.: New Method for Determining Circulation Time Throughout Vascular System, Am. Heart J. 12: 511, 1936.
22. Zwillinger, L.: Ueber die Magnesiumwirkung auf das Herz, Klin. Wchnschr. 14: 1429, 1935.
23. Neurath, O.: Untersuchungen über die Bestimmung der Blutumlaufsgeschwindigkeit mit Magnesiumsulfat, Ztschr. f. klin. Med. 132: 134, 1937.
24. Gubner, R., Schnur, S., and Crawford, J. H.: Use of CO_2 Inhalation as Test of Circulation Time, J. Cl[illegible] 18: 395, 1939.
25. Koch, E.: Die [illegible] des Blutes, Deutsches Arch. f. klin. Med. 104: 39, 1922.
26. Fishbach, D. B.: New Objective Circulation Time Test (Fluorscein Method), J. Lab. & Clin. Med. 26: 1966, 1941.
27. Sperling, L., Weisman, S., and Papermaster, R.: The Effect of Intravenous Theophylline With Ethylene Diamine (Aminophyllin) Upon the Rate and Depth of Respiration, Surgery 11: 600, 1942.

CHRONIC HEMOLYTIC ANEMIA WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA*

Case Report With Experimental Studies

Byron J. Hoffman, M.D., and Roy R. Kracke, M.D.
Emory University, Ga.

CHRONIC hemolytic anemia with the accompanying features of hemoglobinemia and paroxysmal nocturnal hemoglobinuria is characterized by constant and excessive intravascular red blood cell destruction with exacerbations that are provoked by sleep, whether during the day or night. This syndrome was first described by Marchiafava and Nazari in 1911,[1] discussed again in 1928 and 1931 by Marchiafava,[2] and at length in 1931 by Micheli,[3] and is frequently called the Marchiafava-Micheli syndrome. Approximately 40 cases have been reported.

This disease has two fundamental features; first, the intravascular destruction of the erythrocytes, mainly during sleep, and secondly, the paroxysmal appearance of hemoglobin in the urine.

The agent producing this type of intravascular hemolysis remains unknown. The presence of hemoglobin in the blood plasma between the periods of hemoglobinuria, the anemia, if the regenerative efforts of the bone marrow do not fully compensate for the excessive hemolysis, and the persistent reticulocytosis are the signs of constant erythrocytic destruction. There is no familial tendency and no relation to food, drink, posture, drugs, or geographical distribution has been noted. Prolonged exposure to sun, severe chilling, and physical effort cause no exacerbations. About 70 per cent of the cases have been in males, with an average age incidence between 20 and 40 years. In the reported cases the disease has been fatal, usually within two to five years, and all therapeutic procedures, including splenectomy, have been unsuccessful. Mackenzie,[4] in discussing the "march" type of hemoglobinuria observed in soldiers after long marches, or occasionally in persons after severe physical effort, suggested that the increased blood levels of carbon dioxide during exercise may act as a hemolytic agent.

Ham,[5] who has studied this disease very thoroughly in five cases, concluded that the defect was present in the red blood cells, and not in the blood serum, and that the erythrocytes were abnormally sensitive, even within physiologic range, to normal variations of the blood pH toward the acid side during sleep. Ham, as well as Buell and Mettier,[6] demonstrated that the essential factor for hemolysis in the serum of these patients is destroyed by heating to 56° C. and is not restored by the addition of complement. Buell and Mettier,[6] after carrying out studies on the hydrogen-ion concentration, concluded that the fundamental disturbance appears to be in the red blood cells which become sensitized to a lysin normally present in human serum.

*From the Department of Pathology, Emory University School of Medicine, Emory University.

Received for publication, August 25, 1942.

In view of the above concept, alkali salts have been administered by various investigators in an effort to render the blood pH more alkaline, but this has resulted only in temporary improvement, followed by an increase in the degree of hemoglobinemia and hemoglobinuria. Likewise, in the case reported here, this has proved to be an unsatisfactory procedure, since the prolonged administration of alkali salts was followed by a marked accentuation of the hemoglobinemia and hemoglobinuria.

The mechanism responsible for hemoglobin in the urine includes two major factors. The first, as stated before, is the intravascular destruction of the erythrocytes in excess of the reticulo-endothelial capacity to convert the released oxyhemoglobin into bilirubin, thereby resulting in free hemoglobin being dissolved in the blood plasma; and secondly, the renal threshold for hemoglobin. The latter is based upon the functional capacity of the nephron unit to pass the large hemoglobin molecule when an adequate plasma concentration is attained. An explanation of this threshold might be found in Whipple's[7] "glomerular threshold" concept whereby it is assumed that hemoglobin passes through the glomerular filter and is rapidly reabsorbed by the tubular epithelium. As the rate of filtration through the glomeruli exceeds the rate of absorption by the tubules, hemoglobin appears in the urine. It is generally conceded that hemoglobin passes the glomerular filter. Bieter[8] has shown that the aglomerular nephron does not excrete hemoglobin even after being damaged with bichloride of mercury. As yet it is not known how the large hemoglobin molecule with a molecular weight of 67,800 passes the glomerular filter when the molecule of serum albumin with a smaller molecular weight of 67,000 fails to pass it. Bieter,[8] on the basis of variation in size of the glomerular membrane pores, has estimated that 3 per cent of these are of sufficient size for the passage of the hemoglobin molecule. Yet hemoglobin has been observed in the urine of these patients without albumin.

Factors other than molecular size may also be involved in the passage of proteins through the glomerular membrane. Risse[9] maintains that the electrical charge of the protein ion and the glomerular membrane is concerned. Webster[10] and his associates studied the physicochemical factors controlling the passage of proteins across the glomerular membrane and found that the elimination of dissolved hemoglobin by the perfused frog kidney was greatly increased at the acid side (pH 5.5) and was much less at the alkaline side (pH 7.8) of the pH range studied.

If the permeability of the glomerular membrane is altered on exposure to high concentrations of hemoglobin, albuminuria theoretically, at least, should accompany hemoglobinuria, yet it does not always do so. Different threshold values have been observed in human beings and in dogs. Monke and Yuile[11] have determined the average value for the initial rate of tubular absorption in dogs to be between 2 and 3 mg. per minute, with diminishing values to less than 1 mg. per minute after the threshold has been lowered by daily injections of hemoglobin sufficient to cause gross hemoglobinuria.

In summary then, the mechanism may be a combination of altered glomerular permeability and tubular reabsorption, but at the present time most of the evidence indicates that tubular reabsorption capacity is entirely responsible for the phenomenon and hemoglobin will appear in the urine when the plasma concentration attains a level of 130 to 150 mg. per 100 c.c.

CASE REPORT

Present Illness.—The patient dates the onset of his weakness from July, 1935, following the extraction of a tooth, at which time no unusual quantities of blood were lost. Because of this weakness he consulted a physician who informed him that he had pernicious anemia and advised treatment. Liver injections were begun. At about this same time he began to have indigestion which he described as a postprandial epigastric fullness, some abdominal gaseous distention, and a burning sensation which extended from the pharynx to the epigastrium. Some of his friends first commented on his pale appearance during 1934. After a vacation he returned to work in August, 1935, and continued his duties until hospital admission, except for an occasional day or two when he remained at home because of weakness. He was treated constantly by his physician with weekly injections of liver extract, and sometimes from two to three injections per week from 1935 until the first half of 1939. During 1935 he first noticed that his morning specimen of urine was frequently dark red to almost black in color. This continued to a variable degree until June, 1939, when the day urine specimens were sometimes dark and the morning specimen always red to black.

His anemia became so severe in 1938 that he was given his first transfusion. A total of six were given during this year and the first half of 1939. Each time he would return to his work after a few days of rest at home.

Following a transfusion he had a mild chill and noticed that his urine was much darker, but this was not disturbing for he had noticed the same type of discoloration on former occasions. Also, after another citrate transfusion of 500 c.c. of whole blood in November, a severe generalized urticaria and edema of the entire body occurred. These subsided following the administration of adrenalin subcutaneously, ephedrine sulfate by mouth, and several days of bed rest.

Past History.—Childhood illnesses included whooping cough and pneumonia at one year of age, and later mumps and measles without complications. During childhood he played with other children without fatigue. In 1928 he became employed in the box department of a paper company and remained at his job until May 1, 1935, when he was transferred to the stereotype department. This work was done in a poorly ventilated room and consisted of melting the metal composition at very high temperatures and pouring the type.

System Review: The cardiorespiratory, gastrointestinal, and genitourinary systems were negative except as mentioned above. There was no evidence of food allergy. He has never had areas of anesthesia, paresthesia, or hyperesthesia. There was a gradual loss of weight from 160 pounds in 1935 to 140 pounds in 1939.

Family History: His mother died in her late thirties following complications of tubal pregnancy; his father died at 50 years of age with Bright's disease; and one brother and one sister died in infancy from unknown causes. One brother is living and well. Deaths on both sides of the family have usually been due to kidney and heart disease.

Marital History: He married in April, 1935. His wife had always been in good health but never became pregnant as precautionary measures were taken.

Physical Examination.—He was a well developed and moderately well nourished white male, about 27 years of age, with a distinct pallor but no excessive skin pigmentation. His temperature was 98° F., pulse rate 88, respiration normal, and blood pressure 154/74. The positive physical findings were confined to a slight icterus of the sclera and a marked pallor of the conjunctiva and the oral mucous membrane. The gums around the lower central incisors were diseased with pyorrhea and were beginning to recede from the teeth. There was no evidence of cardiac enlargement. Soft blowing systolic murmurs were present at the mitral, aortic, and pulmonic areas. The aortic second sound was loud and snapping and greater than the pulmonic second sound. The liver was normal in size, the spleen was not palpable nor was its area of dullness increased. The reflexes were physiologic and lymphadenopathy was confined to a few small shotty glands in the inguinal region. The nail beds were very pale. The weight was 145 pounds.

Laboratory Findings.—

Erythrocytes	2,170,000 per cubic millimeter
Hemoglobin	7.7 Gm. (50 per cent)

Leucocytes	4,650 per cubic millimeter
Differential count	
Mature segmenters	54 per cent
Bands	14
Eosinophiles	1
Lymphocytes	25
Monocytes	6
Reticulocytes	20 per cent
Platelets	150,000 per cubic millimeter
Clot retraction time	Normal
Bleeding time	Normal
Coagulation time	Normal
Blood group	O (International)
Kahn	Negative
Volume index	1.01
Sedimentation rate	Westergren—70 mm. at one hour
	Wintrobe (corrected)—46 mm. at one hour

The stained smear revealed the erythrocytes to have an average or slightly larger than normal diameter, giving the appearance of slight macrocytosis with a slight degree of hyperchromia accompanied by a moderate variation in size and shape of the red blood cells. There were no abnormal leucocytes, and the platelets were normal in number and appearance. Thick- and thin-drop preparations for malarial parasites were negative. Blood examination during the night for filaria was negative. The van den Bergh reaction was indirect with 1.7 mg. of bilirubin per 100 c.c. of serum.

Icterus index	17	
Blood cultures	Anaerobic, aerobic, and under 10 per cent carbon dioxide were negative	
Blood chemistry		
Nonprotein nitrogen	32 mg. per 100 c.c.	
Creatinine	1.3 mg. per 100 c.c.	
Sugar (fasting)	106 mg. per 100 c.c.	
Calcium	11.6 mg. per 100 c.c.	
Phosphorus	3.6 mg. per 100 c.c.	
Chlorides (whole blood)	480 mg. per 100 c.c.	
Sodium (serum)	376 mg. per 100 c.c.	
Potassium (serum)	20 mg. per 100 c.c.	
Cholesterol	126 mg. per 100 c.c.	
Total protein	6.9 Gm.	
Albumin	4.0 Gm.	
Globulin	2.9 Gm.	
Fragility test	Control	Patient
Beginning hemolysis	42	42
Complete hemolysis	32	32

Spectroscopic examination of plasma and serum revealed absorption bands of oxyhemoglobin. No porphyrin or other abnormal bands were present.

Morning urine	
Color	Dark red
Specific gravity	1.025
Albumin	Trace
Acetone	Negative
Diacetic acid	Negative
Benzidine test	4 plus
Bile	Negative

Urobilinogen	Slightly increased. Morning specimen positive in 1:30 and day specimen 1:20 dilutions
Microscopic	Innumerable triple phosphate and amorphous phosphate crystals. No red cells
Spectroscopic	Revealed absorption bands of oxyhemoglobin. No prophyrin absorption bands were present

All specimens of urine voided during the day and night were saved. Without exception those voided during the night and on rising were red to black in color without the presence of red blood cells and gave a strongly positive benzidine reaction. The patient's sleeping habits were reversed so that he slept during the day and stayed up at night. Still the red to black urine followed sleep, whether during the day or night.

Donath-Landsteiner Chilling Test	Negative
Urine examination for lead	0.035 mg. per 100 c.c.—patient 0.035 mg. per 100 c.c.—control
Cevitamic acid content of urine per twenty-four hours	20 mg.

Cystoscopic examination and retrograde pyelograms were normal.

The kidneys excreted 67 per cent of the phenolsulfonphthalein dye in one hour after it was given.

Skin biopsy (stained with Prussian blue)	Negative for hemosiderin
Basal metabolic rate	Plus 8
Stool examination	There were no parasites, ova, or abnormal bacterial flora on culture studies.
Gastric analysis	
Fasting specimen	35 degrees free acid
30 minutes after Ewald meal	52 degrees free acid
Lactic acid	Negative
Blood and other abnormal contents	Negative

Skin tests for food sensitivity: Positive reactions were obtained for shrimp, scallop, crab, cherry, prune, cocoa, banana, carrots, celery, mushroom, cranberry, and dates.

Diagnosis.—The diagnosis of paroxysmal nocturnal hemoglobinuria was based on the nocturnal exacerbations of hemoglobinuria associated with the anemia, reticulocytosis, and hemoglobinemia. Paroxysmal cold hemoglobinuria, the most common of the paroxysmal types, was excluded since there was no history or clinical evidence of syphilis; the Wassermann, Kahn, and Donath-Landsteiner reactions were negative, and exacerbations of hemoglobinuria were not provoked by chilling.

Paroxysmal march hemoglobinuria was not considered, since it is usually observed in soldiers following long marches and is directly related in other isolated instances to physical effort. In the march type there is no evidence of a constant intravascular erythrocytic destruction, and even though there is a transient hemoglobinemia there is no sustained anemia, reticulocytosis, or icterus.

Favism,[12] a form of allergic paroxysmal hemoglobinuria, was excluded by the elimination without beneficial results of those foods to which the patient gave positive skin reactions.

EXPERIMENTAL STUDIES

Blood pH Determinations.—Procedure: Venous blood was drawn from the anterior cubital vein into a dry syringe without the use of a tourniquet. Heparin was used as the anticoagulant, and no suction was applied to the plunger of the syringe during the withdrawal of the blood. The Beckman glass electrode potentiometer was used to obtain the hydrogen-ion determination. After withdrawal the blood was immediately transferred to the cup of the instrument, the glass electrode was immersed, and the pH was determined. Controls were done on normal persons using the same instrument and technique. All blood specimens taken during waking hours were obtained after two or more hours of bed rest. Blood samples were taken immediately after being awakened in the morning, in the evening, and during the night hours (see Table I). The patient and control were awakened from sleep for the latter. In one instance the sleeping habits of the patient and control were reversed and determinations were made.

TABLE I

DATE	HOUR	pH OF VENOUS BLOOD		PATIENT'S PLASMA Hb.	COLOR OF PATIENT'S URINE	BENZIDINE REACTION OF URINE
		PATIENT	CONTROL	CONC. IN MG./100 C.C.*		
12/ 7/39	7:00 P.M. (up all day)	7:37	7:39	80	Yellow	-
12/ 7/39	12:00 P.M. (awakened from sleep)	7:40	7:43	180	Dark amber	+
12/ 8/39	7:00 P.M. (up all day)	7:29	7:32	110	Amber	-
12/ 9/39	7:30 A.M. (awakened)	7:33	7:30	280	Black	++++
12/10/39	12:00 P.M. (awakened)	7:43	7:24	270	Black	+++
12/11/39	7:00 P.M. (up all day)	7:43	7:32	120	Yellow	-
12/12/39	8:00 P.M. (up all day)	7:30	7:35	95	Yellow	-
12/13/39	8:00 A.M. (up all night)	7:39	7:35	105	Yellow	-
12/13/39	5:00 P.M. (slept during day)	7:39	7:31	185	Dark red	++
12/14/39	7: A.M. (up all night)	7:35	7:30	100	Yellow	-
12/14/39	1:30 P.M. (awakened from sleep)	7:45	7:42	181	Dark red	++

*Blood plasma of control contained no hemoglobin.

No consistent variations of the blood hydrogen-ion concentration, as indicated by blood pH determinations, could be shown on the specimen of blood taken after the patient was up the entire day or on the specimen obtained when the patient was awakened from sleep, whether during the night or at the usual

hour of rising. The mean average for pH determinations obtained after being awake was 7.35 and that after being asleep was 7.40; whereas that of the control subject during waking hours was 7.33 and after being asleep was 7.34.

The observations on this patient and the control showed very little difference in the pH of the blood after being awake and on being awakened from sleep. The mean values obtained after sleep are slightly more alkaline in both the patient and the control. In this case, there does not appear to be a definite relationship between the hydrogen-ion concentration of the plasma and the nocturnal exacerbations of the intravascular red blood cell destruction.

As Ham has pointed out,[5] it is possible that the deeper portions of the body have a greater variation of the blood pH, within the normal range, on the physiologic basis of a slowed blood stream and shallow respiration during sleep. However, it would appear that the slowed blood stream and shallow respirations during sleep are evidences of a lowered metabolism accompanying a decrease in the physiologic demands of the respiratory and circulatory systems at this time. Furthermore, the reserves of the blood buffer system should be adequate to compensate for slight variations of carbon dioxide during sleep without disturbances of the blood pH if it is capable of maintaining the balance during exercise.

Plasma Hemoglobin Determinations.—The blood plasma was utilized for quantitative plasma hemoglobin determinations by using the Sheard-Sanford photelometer. Comparatively large volumes of plasma (from 2 to 3 c.c.) were necessary to obtain accurate and consistent readings. A voided specimen of urine was obtained from the patient each time blood samples were drawn. The color and the benzidine reaction of each are recorded in Table I.

The plasma hemoglobin concentration obtained during different times of the day and night varied from 80 to 280 mg. per 100 c.c. of plasma. The lower levels occurred after being awake for a number of hours and the higher levels following sleep.

The urine specimens varied in color and intensity of the benzidine reaction. All the blood plasma concentrations between 80 and 120 mg. per 100 c.c. had an accompanying yellow or amber urine which did not contain hemoglobin, whereas the levels from 180 to 280 mg. per 100 c.c. had a urine red to black in color and a marked variation in the intensity of the benzidine reactions. No blood levels were obtained between 120 and 180 mg. per 100 c.c.

The urines were usually red and gave a 2 plus benzidine reaction when the plasma levels of hemoglobin were 180 mg. per 100 c.c. This would indicate, as near as can be determined from voided specimens, that the renal threshold for hemoglobin in this person is well below 180 mg. per 100 c.c., and above 120 mg. per 100 c.c.

Effect of Sympathetic Stimulant.—As sleep in this disease is accompanied by exacerbations of intravascular erythrocytic destruction, the physiologic features concerning the state of sleep were considered. The cause and nature of sleep remain obscure. Many theories have been advanced. Among the more widely accepted is that of Hess,[13, 14] who after extensive investigation concluded that sleep was a parasympathetic function. He pointed out in support of his theory that the constriction of the pupils, the bradycardia, and the vasodilatation

accompanying sleep are parasympathetic functions and respond to parasympathetic stimulants. On this basis, a series of experimental studies were carried out on this patient in an effort to determine the effects of sympathetic stimulants.

TABLE II

DATE	TIME AND AMOUNT DRUG GIVEN	URINE COLOR AND TIME VOIDED		COLOR OF SPECIMEN REMAINDER OF DAY	R.B.C.	HB. (GM.)	RETIC. (%)
1/25/40	None	Red 2:30 A.M. Benzidine positive	Black 8:00 A.M. Benzidine positive	Yellow to amber with occasional benzidine positive	2,100,000	7.8	18
1/26/40	0.5 c.c. adrenalin 9:00 P.M. and 1:00 A.M.	Light amber 1:00 A.M. Benzidine positive	Amber 7:00 A.M. Benzidine positive	Same	2,100,000	7.8	14
1/27/40	0.5 c.c. adrenalin 9:00 P.M. 2:00 A.M.	Yellow 2:00 A.M. Benzidine positive	Light red 7:00 A.M. Benzidine positive	Same	2,100,000	7.8	14
1/28/40 through 1/31/42	0.5 c.c. adrenalin 9:00 P.M. 2:00 A.M.	Dark amber 2:00 A.M. Benzidine positive	Amber 8:00 A.M. Benzidine positive	Same	2,260,000 2,240,000 2,470,000	8.3 8.5 8.5	12 12 14
2/ 1/40 2/ 2/40	Adrenalin omitted	Light red 4:00 A.M. Benzidine strongly positive	Light red 8:00 A.M. Benzidine strongly positive	Same	2,300,000	8.3	14
2/ 3/40 through 2/14/40	0.5 c.c. adrenalin 9:00 P.M. 2:00 A.M.	Yellow to amber 2:00 A.M. Occasional benzidine positive	Amber 8:00 A.M. Occasional positive benzidine	Same	2,250,000	8.6	14

The first was adrenalin. One-half cubic centimeter doses of adrenalin in oil (Parke, Davis & Co., 1:1,000 concentration) were administered subcutaneously at 9:00 P.M. and 3:00 A.M. each night (Table II). The date (Jan. 25, 1940) listed on Table II as having red to black urine after sleep is typical of the nocturnal hemoglobinuria occurring at that time. On the nights following the subcutaneous administration of adrenalin only an occasional urine contained sufficient hemoglobin to cause red discoloration. The injections were omitted on the nights Feb. 1, 1940, and Feb. 2, 1940, and the urines again were red with hemoglobin following sleep. The administration of adrenalin was resumed, and the urines returned to a yellow or amber color with only an occasional morning specimen having a positive benzidine reaction. The red blood cell count, hemoglobin, and degree of reticulocytosis were not appreciably altered during this time. The erythrocyte count increased about 400,000 cells per cubic millimeter and did not increase further even though the degree of nocturnal hemoglobinuria had decreased and the patient felt much better. The whole blood hemoglobin increased only slightly while the regenerative activity, as indicated by the degree of reticulocytosis, remained constant.

Synthetic suprarenin was then substituted for the aqueous extract of the suprarenal gland from Feb. 14, 1940, through Feb. 19, 1940. Beginning Feb. 16, 1942, the morning urines varied from a dark amber to very light red color, and all specimens after sleep gave a strongly positive benzidine reaction. During this period the red blood cell count remained at 2,300,000 cells per cubic millimeter.

All medication was discontinued on Feb. 19, 1940. After several days an occasional specimen voided during the night and the morning specimen of urine varied from a light to dark red color. Curiously enough, the synthetic suprarenin, in the same dosage and concentrations, did not bring about the same improvement in the hemoglobinuria as the aqueous extract of the medullary portion of the suprarenal gland. This would suggest the presence of additional beneficial agents in the natural extract other than adrenalin.

Effect of Parasympathetic Stimulants.—Since a sympathetic stimulant, adrenalin, had improved the state of hemoglobinuria and hemoglobinemia without appreciably altering the status of the blood, it seemed worth while to determine the effect, if any, of parasympathetic stimulants.

As an accentuation of the hemolysis and subsequent exacerbation of hemoglobinuria seemed possible, small doses of prostigmine (1 c.c. of a 1:4,000 solution) were given subcutaneously at the usual hours of 9:00 P.M. and 3:00 A.M. All urine specimens became normal in color, with only an occasional one giving a positive benzidine reaction. Injections of prostigmine were continued for seven nights. Then eserine sulfate in a dosage of 0.7 mg. subcutaneously at the same hours of 9:00 P.M. and 3:00 A.M. gave the same results. Pilocarpine hydrochloride in 3 mg. doses subcutaneously was started on March 11, 1940, and continued through March 26, 1940. All urines remained yellow to amber in color, free of hemoglobin, and the patient felt much better during this period of time. However, the red blood cell count never went above the level of 2,500,000 with 7.6 Gm. of hemoglobin. All urine specimens were of a normal color, with an occasional one giving a positive reaction for hemoglobin. Pilocarpine hydrochloride had been discontinued for twenty days before the urines voided during the night and on arising in the morning again became dark amber in color and consistently gave positive benzidine reactions. The red blood cell count at this time varied from 1,800,000 to 1,950,000, approximately 400,000 less than the levels obtained during the administration of parasympathetic stimulants.

Eschatin, the aqueous extract of the adrenal cortex prepared by Parke, Davis & Co., was started on April 15, 1940, in 0.5 c.c. doses subcutaneously at 9:00 P.M. and 3:00 A.M. and continued through April 25, 1942. This preparation was used because of the beneficial results obtained with the natural adrenalin, whereas the use of synthetically prepared epinephrine appeared to be of little or no value. The urines returned to a light amber or yellow color and were free of hemoglobin. After several days the red blood cell count increased to 2,300,000 cells per cubic millimeter but never attained higher levels.

Ten grams of sodium chloride were then administered orally in conjunction with the eschatin for a period of a week without beneficial or harmful effects. Then synthetic cortin, desoxycorticosterone, was substituted for eschatin during

a period of hemoglobinuria without alteration in the degree of the nocturnal hemoglobinuria.

The urines continued to be free of hemoglobin throughout the months of May and June, the erythrocyte count remained at about 2,200,000 cells per cubic millimeter, and the reticulocyte count varied from 10 to 20 per cent. At this time the patient was dismissed from the hospital, advised to limit his physical activities, and return for observation in the out-patient department.

Subsequent Course.—The patient did as he was advised and maintained an erythrocyte count of 2,100,000 with 21 per cent reticulocytes for the following six months. In January, 1941, he contracted influenza, and was hospitalized, and treated symptomatically. At that time the erythrocyte count was 1,680,000 per cubic millimeter with 12 per cent reticulocytes, but returned to 2,000,000 per cubic millimeter a few days after the infection disappeared. No hemoglobinuria occurred during this time. The temperature of the patient was always normal except during the upper respiratory infection. At this time the condition of the patient is about the same.

GENERAL DISCUSSION

The results of the agents employed in these studies are offered without an explanation of their mechanism. Papilian and Antonescu-Mazilu[15] recently investigated the antihemolytic action of adrenalin and showed that natural adrenalin inhibits the hemolytic effect of mercuric chloride and digitalis on erythrocytes in vitro and in animals. It is unusual that the aqueous extracts of the medullary and cortical portions of the adrenal gland will have similar effects on the nocturnal exacerbations of erythrocytic destruction. Yet the synthetic preparations of adrenalin and cortin were for the most part ineffective. This would suggest that the antihemolytic agent is neither adrenalin nor cortin, but another product which is present in both portions of the adrenal gland and is water soluble. Eschatin, the natural cortical extract, was more efficacious in decreasing the nocturnal exacerbations of hemolysis without having the stimulating side effects of adrenalin. Polycythemia[16] with characteristics of polycythemia vera has been observed in adrenal cortical tumors and basophilic adenomas of the pituitary.

The action of the parasympathetic stimulants is equally obscure. Stimulation of the parasympathetic system serves as an activator to the secretory epithelium of the gastrointestinal tract, its appendages, and the sweat glands of the integument. Perhaps the parasympathetic also serves to stimulate the adrenal gland to produce an antihemolytic agent or some product concerned with the development of erythrocytes that are more resistant to normal blood hemolysins.

These agents sufficiently decreased the hemolysis during the usual nocturnal exacerbations to lower the plasma hemoglobin below the renal threshold levels, thereby eliminating the symptom of hemoglobinuria which should be regarded as no more than a urinary manifestation of excessive intravascular red blood cell destruction.

It is not likely that the agents used in these studies acted favorably by altering the renal threshold for hemoglobin. Numerous physicochemical factors have been cited as controlling the passage of proteins across the glomerular membrane. Krogh[17] reviewed the evidence that the increased permeability of

the glomerular membrane often paralleled an increase in capillary diameter. Webster[10] and his associates, in their work on the perfusion of hemoglobin by the frog's kidney, found the glomerular membrane more permeable to hemoglobin and the capillary bed dilated at the acid end of the pH range. However, in the studies cited in this paper the hemoglobinuria was greatly lessened by capillary constrictors and disappeared with capillary dilators. Perhaps the improvement in the hemoglobinuria observed by Ham[5] following a limited administration of alkali salts and the relapse after excessive quantities results from an alteration in the physicochemical factors of the capillary bed of the glomeruli rather than the degree of intravascular red blood cell destruction.

It is impossible to know how much, if any, the course of the disease process has been altered in this patient by decreasing the nocturnal exacerbations of the hemolysis since the state of anemia has not been appreciably altered. Splenectomy has been reported to lessen the severity of the nocturnal hemoglobinuria, but the state of anemia and the downward course of the disease continued unaltered in these cases.

SUMMARY

1. A case of chronic hemolytic anemia with paroxysmal hemoglobinuria (Marchiafava-Micheli syndrome) is reported.

2. Reversal of the sleeping habits of the patient provoked hemoglobinuria during sleep, whether during day or night.

3. Determinations of the blood pH did not reveal abnormal variations in the hydrogen-ion concentration after the patient was awake all day or after sleeping all night. The mean average after sleep was slightly more alkaline (pH 7.4) than after being awake (pH 7.35).

4. The renal threshold for hemoglobin in this patient was between 120 and 180 mg. per 100 c.c.

5. The severity of nocturnal exacerbations of intravascular red blood cell destruction was markedly lessened by the use of adrenalin and eschatin, the aqueous extracts of the medullary and cortical portions of the suprarenal gland. Synthetic adrenalin and desoxycorticosterone did not alter the nocturnal exacerbations of hemoglobinuria.

6. The parasympathetic stimulants prostigmine, eserine sulfate, and pilocarpine hydrochloride were more efficacious than the suprarenal extracts in lessening the degree of the nocturnal red blood cell destruction and subsequent exacerbations of hemoglobinuria.

7. Pilocarpine hydrochloride was the most effective. On each occasion after its use hemoglobin disappeared from the urine for many days or even weeks.

8. The degree of anemia and reticulocytosis were not appreciably altered during the remissions of hemoglobinuria that were produced by the various agents employed in these experimental studies.

REFERENCES

1. Marchiafava, E., and Nazari, A.: Nuova contributa allo studio degli itteri chronici emolitici, Policlinico (sez. med.) 18: 241, 1911.
2. Marchiafava, E.: Anemia emolitica con emosiderinuria perpetua, Policlinico (sez. med.) 35: 109, 1928; 38: 105, 1931.

3. Micheli, F.: Anemia (splenomegalia) emolitica con emoglobinuria-emosiderinuria tipo Marchiafava, Haematologica, I. Arch. 12: 101, 1931.
4. Mackenzie, G. M.: Paroxysmal Hemoglobinuria, Review, Medicine 8: 159, 1929.
5. Ham, T. H.: Studies on Destruction of Red Blood Cells. Chronic Hemolytic Anemia With Paroxysmal Nocturnal Hemoglobinuria. An Investigation of the Mechanism of Hemolysis, With Observations on Five Cases, Arch. Int. Med. 64: 1271, 1939.
6. Buell, A., and Mettier, S. R.: Paroxysmal Nocturnal Hemoglobinuria With Hemolytic Anemia (Marchiafava-Micheli Syndrome), J. Lab. & Clin. Med. 26: 1434, 1941.
7. Lichty, J. A., Jr., Havill, W. H., and Whipple, G. H.: Renal Thresholds for Hemoglobin in Dogs, J. Exper. Med. 55: 603, 1932.
8. Bieter, R. N.: The Action of Some Diuretics Upon the Aglomerular Kidney, J. Pharmacol. & Exper. Therap. 43: 386, 1931.
9. Risse, O.: Permeability of Collodion Film and of Albuminous Membranes for Certain Ampholytes; Influence of H and OH Ion Concentration, Arch. f. d. ges. Physiol. 212: 375, 1926.
10. Webster, M. D., Engel, F. L., Laug, E. P., and Amberson, W. R.: Influence of pH Upon Elimination of Hemoglobin by the Perfused Frog's Kidney, J. Cell. & Comp. Physiol. 5: 399, 1934.
11. Monke, J. V., and Yuile, C. L.: The Renal Clearance of Hemoglobin in the Dog. J. Exper. Med. 72: 149, 1940.
12. Kracke, R. R.: Diseases of the Blood, ed. 2, Philadelphia, 1941, J. B. Lippincott Co.
13. Hess, W. R.: The Autonomic Nervous System, Lancet 2: 1199, 1932.
14. Hess, W. R.: The Mechanism of Sleep, Am. J. Physiol. 90: 386, 1929.
15. Papilian, V., and Antonescu-Mazilu, F.: Antihemolytic Power of Epinephrine, Cluj. med. 21: 123, 1940.
16. Kepler, E. J., and Keating, F. R.: Diseases of the Adrenal Glands. Tumor of the Adrenal Cortex, Diseases of the Adrenal Medulla and Allied Disturbances, Arch. Int. Med. 68: 1010, 1941.
17. Krogh, A.: The Anatomy and Physiology of the Capillaries, New Haven, Conn., 1929, Yale University Press.

VARIATIONS IN SUSCEPTIBILITY TO CINCHOPHEN AS OBSERVED IN ANIMALS WITH BILE FISTULAS*

J. H. Annegers, M.S., F. E. Snapp, M.S., A. J. Atkinson, M.S., M.D., and A. C. Ivy, Ph.D., M.D., Chicago, Ill.

It has been suggested by clinical and experimental observers that in some instances, if not all, the toxic manifestations of cinchophen are due to hypersensitivity.[1]

In the course of our studies of the elimination of cinchophen in the bile of dogs with chronic biliary fistulas, observations have been made which show that some dogs are more sensitive than others and that some dogs react more markedly to the first dose of the drug than to subsequent doses.

In this study we have directed our attention to disturbances of cholic acid output in the bile as well as to such symptoms as anorexia, vomiting, and diarrhea. It should be stated in advance that the animals used in these experiments represent a selected group to establish variations in susceptibility in the same animal and in different animals with a bile fistula. The seven susceptible ani-

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

Received for publication, Aug. 26, 1942.

*Acknowledgment is gratefully made to the E. L. Dawes and Marjorie Newman Funds for financial assistance in this project.

mals to be referred to come from a group of dogs with biliary fistulas which were given cinchophen several times over periods varying from two to thirty-three days.

METHODS

The animals had a chronic bile fistula of the Rous-McMaster type;[2] in addition, some had a duodenal fistula for the return of bile into the intestine. The animals weighed from 7 to 13 kg. All were allowed to recover from the operation and to reach a constant twenty-four-hour output of cholic acid on the standard diet. Numerous control tests on the diet with and without the return of bile had been conducted prior to the administration of cinchophen.

Three different types of experiments were performed. In those of Group I, the initial dose of cinchophen was given after a period of from three to four days, during which time the animal reached a basal control output of cholic acid on the diet fed twice daily without the return of bile. The cholic acid so formed comes from synthesis only. In those in Group II the daily ration of the standard diet was divided into three portions and fed every eight hours, with the bile secreted during the previous eight hours being returned to the duodenum during a period of about one hour after the meal. When the output of cholic acid had reached a relatively steady level, 0.333 Gm. of cinchophen was given with each meal. Thus the bile salts and cinchophen excreted in the bile (45 to 55 per cent of the oral dose) were subjected to three enterohepatic circulations daily. In the experiments in Group III, the animals, after being subjected to a basal control period on the diet without the return of bile, were given through the duodenal fistula the bile secreted during the preceding hour. At the start of the hourly return of bile, 0.5 Gm. of cinchophen was given with the meal, and another dose of 0.5 Gm. was given with the next meal twelve hours later, and thenceforth until 2 Gm. were given, the hourly return of bile being continued. Thus, the bile salts and cinchophen excreted in the bile were subjected to an enterohepatic circulation rather continuously as would occur in a patient without a gall bladder or with a nonfunctioning gall bladder.

The dose of cinchophen for the human adult varies from 2 to 5 Gm. daily, or from 30 to 70 mg. per kilogram. The average dose received by our animals was approximately 100 mg. per kilogram, or on the basis of body weight it was equivalent to twice the average dose given to man. A snow-white cinchophen powder* was used, which was odorless and completely soluble in alkaline aqueous solution.

RESULTS

Group I.—The four dogs referred to in Table I were allowed to reach their basal output of cholic acid on the diet. Then 0.5 Gm. of cinchophen was given with the meal every twelve hours for from three to five days.

One of the dogs B8, on the second day of medication (Table I) started and continued to vomit during the two-hour period preceding each meal. Since her stomach was about empty at the time, little food was lost. This is important because vomiting, particularly of food, decreases cholic acid output, since much

*Calco Chemical Co.

of the cholic acid is synthesized from the protein of the ingested food. After withdrawal of the cinchophen, the cholic acid output returned to the normal level within three days, during which time no vomiting occurred. Later, this animal was subjected several times to the same dose of cinchophen: no vomiting or anorexia and no decrease in cholic acid output occurred.

Table I

A Dose of 0.5 Gm. of Cinchophen Was Given With the Meals Twice Daily Without the Return of Bile

DOG	DATE	BILE VOL. C.C.	TOTAL CHOLIC ACID (MG. PER 24 HR.)	REGIME	
BS	Control	109	1,226	Control; average of six days	
	2/24	234	1,508	0.5 Gm. cinchophen b.i.d.	
	2/25	218	873	0.5 Gm. cinchophen b.i.d.	Vomited a little
	2/26	232	684	0.5 Gm. cinchophen b.i.d.	before meals
	2/27	202	738	0.5 Gm. cinchophen b.i.d.	
CS	Control	136	1,675	Control; average of three days	
	2/1	325	2,090	0.5 Gm. cinchophen b.i.d.	
	2/2	336	1,686	0.5 Gm. cinchophen b.i.d.	
	2/3	347	1,822	0.5 Gm. cinchophen b.i.d.	
	2/4	310	1,792	0.5 Gm. cinchophen b.i.d.	
C10	Control	150	1,740	Control; average of three days	
	2/6	240	1,620	0.5 Gm. cinchophen b.i.d.	
	2/7	234	1,640	0.5 Gm. cinchophen b.i.d.	
	2/8	249	1,620	0.5 Gm. cinchophen b.i.d.	
C7	Control	138	2,172	Control; average of three days unusually high output of cholic acid for control	
	2/5	262	1,892	0.5 Gm. cinchophen b.i.d.	
	2/6	203	1,386	0.5 Gm. cinchophen b.i.d.	
	2/7	227	1,118	0.5 Gm. cinchophen b.i.d.	
	2/8	336	1,688	0.5 Gm. cinchophen b.i.d.	
	2/9	326	1,923	0.5 Gm. cinchophen b.i.d.	
		The cinchophen was continued for twelve days with normal outputs of cholic acid, then withdrawn and started again three days later			
	2/20	276	488		
	2/21	311	928		
		The cinchophen was discontinued for two weeks and then it was given again for three days with the following results:			
	Control	181	2,280	Control period of 3 days	
	3/5	299	2,590	0.5 Gm. cinchophen b.i.d.	
	3/6	288	2,300	0.5 Gm. cinchophen b.i.d.	
	3/7	247	1,950	0.5 Gm. cinchophen b.i.d.	

Dog C7 (Table I) showed a similar depression of cholic acid output during the second and third days of cinchophen medication which returned to the "normal" level for this dog during the fifth day of medication. This animal manifested no anorexia or vomiting. The cinchophen was continued for a total of twelve days. Then the cinchophen after three days of withdrawal was resumed again. The cholic acid output markedly decreased, though there was no vomiting and no diarrhea. The drug was stopped for two weeks and then given for three days. Some, though not a significant, depression of the cholic acid output occurred on the third day. This dog represents a case in which the cinchophen caused a depression of cholic acid output each time it was given, more the second time it was given than the first. In addition, the cholic acid output was depressed without any apparent gastrointestinal symptoms.

Dogs C8 and C10 are included to illustrate that similar doses of cinchophen have no effect on appetite and cholic acid synthesis in some dogs.

Group II.—The animals in this group were fed every eight hours and received via the duodenal fistula after meals all the bile secreted during the previous eight hours, except 0.1 c.c. which was used for cholic acid determination. After the animals had reached a relatively steady state, 0.333 Gm. of cinchophen was given with each meal. The cinchophen excreted in the bile during eight hours after oral administration amounts to from 45 to 55 per cent of the oral dose. Consequently the liver of these dogs was exposed to about 1.5 Gm. of cinchophen each day. With the exception of the return of bile and the enterohepatic circulation of cinchophen, these experiments were analogous to those in Group I.

Dog C3 vomited the first day of cinchophen administration. After having received 1 Gm. of cinchophen, the medication was stopped, and then the bile return was stopped to ascertain whether the basal control output of cholic acid without the return of bile would be resumed. The data in Table II shows that although cinchophen caused vomiting, it had no definite effect on basal cholic acid synthesis on the diet, since the postcinchophen output of cholic acid was normal.

TABLE II

A DOSE OF 0.333 GM. OF CINCHOPHEN EVERY EIGHT HOURS WITH MEALS; THE BILE WAS RETURNED EVERY EIGHT HOURS JUST AFTER THE MEALS

DOG	DATE	BILE VOL. C.C.	TOTAL CHOLIC ACID (MG. PER 8 HR.)	REGIME
C3	Control	157	1,434	No bile returned, basal control
	8	218	5,002	Bile returned t.i.d.
	9	248	4,927	Bile returned t.i.d.
	10	353	6,332	Bile returned t.i.d., + 0.333 cinchophen; dog vomited once
	11	224	3,951	Bile returned first eight hours only
	12	251	1,475	No bile returned
C4	19	232	4,535	Bile returned t.i.d.
	20	246	5,511	Bile returned t.i.d.
	21	212	5,173	Bile returned t.i.d.
	23	268	2,847	Bile returned t.i.d., + 0.333 cinchophen; anorexia
	24	363	4,705	Bile returned t.i.d., + 0.333 cinchophen; anorexia
				(Other data omitted from Table. See text)
C2	8	212	5,856	Bile returned t.i.d.
	9	223	5,725	Bile returned t.i.d.
	10	365	7,895	Bile + 0.333 Gm. cinchophen t.i.d.
	11	340	6,557	Bile + 0.333 Gm. cinchophen t.i.d.
				(Other data omitted from Table. See text)

Dog C4 showed a significant (44 per cent) depression of cholic acid output during the first day of cinchophen administration. But on continued cinchophen administration, the output of cholic acid remained around 4.7 to 5.0 Gm. per day. The animal had a poor appetite but ingested the daily ration throughout, even during the first day when the output of cholic acid was depressed.

Dog C2 is included to illustrate an animal that showed no effect from the same dose of cinchophen.

Group III.—The animals in this test represent a group in which the bile was returned to the intestine hourly. 1. The animals were placed on the diet with

a meal every twelve hours without the return of bile (Column 1, Table III) until they reached a relatively steady output of cholic acid. 2. The bile secreted the previous hour was returned for a period of forty-eight hours, the cholic acid output for the forty-eight hours being determined by sampling the hourly specimens (Column 2, Table III). 3. Step 1, above, was repeated. 4. Step 2 was repeated except that 0.5 to 0.25 Gm. of cinchophen was given with each meal for forty-eight hours. 5. Step 1, above, was repeated.

TABLE III

A DOSE OF 0.25 OR 0.5 GM. OF CINCHOPHEN WAS GIVEN TWICE DAILY AND BILE WAS RETURNED HOURLY FOR FORTY-EIGHT HOURS; THE TOTAL CHOLIC ACID OUTPUT FOR SEVENTY-TWO HOURS IS GIVEN

DOG	TOTAL MG. CHOLIC ACID FOR 72 HOURS				REMARKS
	AVERAGE BEFORE TEST—NO BILE (24 HR.)	CONTROL TEST WITH BILE	CINCHOPHEN TEST WITH BILE	AFTER CINCOPHEN—NO BILE (24 HR.)	
A3	1,462	22,104	23,011	1,613	0.5 Gm. cinchophen b.i.d.
B3	1,547	--	9,109	910	0.5 Gm. cinchophen b.i.d.; dog died
D4	1,950	7,216	4,822	2,500	0.25 Gm. cinchophen b.i.d.
	1,630*	7,216	9,729	1,701	0.5 Gm. cinchophen b.i.d.; three weeks later
D6	1,023	8,577	5,731	1,252	0.5 Gm. cinchophen b.i.d.
	1,315*	8,577	8,997	950	0.25 Gm. cinchophen b.i.d.; two weeks later
	950*	8,577	7,416	1,060	0.5 Gm. cinchophen b.i.d.; three weeks later
D7	2,360	6,640	9,226	2,710	0.5 Gm. cinchophen b.i.d.

*These later tests are included to show the different cholic acid response to later doses of cinchophen.

Under the preceding plan the cinchophen excreted in the bile was subjected to repeated enterohepatic circulation. The dogs receiving 0.5 Gm. with each meal had their livers exposed to about 2 Gm. of cinchophen daily and the dog which received 0.25 Gm. with each meal had its liver exposed to about 1 Gm. daily. Thus it was possible to compare the effect of the cinchophen on bile formation when bile was being secreted at a rapid rate and the liver exposed to cinchophen constantly and to observe any after effects the cinchophen might have on basal cholic acid synthesis.

Dogs A3 and D7 were not affected by the cinchophen and are included to illustrate that some dogs show no ill effect from this dose and repeated enterohepatic circulation of cinchophen.

Dog B3, however, was markedly affected. It became sick during the second day and ate no food after the first day. The experiment was continued during the second day to ascertain if recovery would ensue. Cholic acid was completely depressed during the first twenty-four hours after cinchophen was stopped; that is, after four doses during forty-eight hours. After that, the cholic acid synthesis returned to about 50 per cent of the control level, in the presence of complete anorexia, and the animal died after having received only 2 Gm. of cinchophen. This animal died of some condition apparently caused by the cinchophen which was not significantly revealed by the mechanism re-

sponsible for cholic acid synthesis. Autopsy revealed only a severe gastroenteritis.

Dog D4 showed a 33 per cent depression of cholic acid output during the first period of administration of 0.5 Gm. cinchophen daily with return of bile and no depression of cholic acid synthesis after the cessation of cinchophen and the return of bile. However, at a subsequent period twice the dose of cinchophen had no apparent effect on the dog.

Dog D6 reacted like Dog D4 to the first period of administration of cinchophen and manifested no significant disturbance of cholic acid output during two subsequent periods, though a slight depression occurred with the second dose of 1 Gm. daily.

DISCUSSION

The variations in susceptibility of different animals to cinchophen has been observed by all who have used it for producing "peptic" ulcers in dogs and for studying its toxicity. We have observed the same variation in susceptibility in our dogs with bile fistulas.

We have also noted that the susceptibility is usually definitely less on the second and later administrations of the drug.

The symptoms in our dogs with bile fistulas were chiefly gastrointestinal (anorexia, vomiting, and diarrhea). This coincides with the observation made by Stalker, Bollman, and Mann,[3] Reid and Ivy,[4] and others,[5] who also observed that on continuous daily administration for several weeks the gastrointestinal symptoms frequently subsided after the first or second week, indicating an increase in alimentary tolerance. Death occurs early in those in which the gastrointestinal disturbance does not subside. Clinically it has been recommended that administration of the drug should cease if gastric distress occurs.[5]

The disturbance in cholic acid output observed in the animals reported in this paper can be accounted for by a gastrointestinal irritation, except in one instance out of seven. Any disturbance of protein digestion and absorption, since all the evidence indicates that cholic acid is synthesized from protein cleavage products, would decrease cholic acid production. Hepatitis of sufficient grade to cause a complete suppression of cholic acid synthesis occurred in only one dog (B3) and then only for one day, the dog succumbing to a severe gastroenteritis. It is possible, however, for cinchophen to disturb materially some important function of the liver which is not reflected by the rate of cholic acid synthesis. But, it is noteworthy that the rate of secretion of cholic acid was not materially affected, as shown by the dogs in Table III, when the liver is secreting bile salts at a level close to its maximum capacity.

We were disappointed in not observing a more definite and unequivocal hepatic injury as indicated by a reduction in cholic acid output in the susceptible dogs. The failure to obtain such evidence of hepatic injury, however, is consonant with the failure to find evidence of microscopic damage of the liver of dogs except when very large doses of cinchophen are given. No one has obtained any evidence of liver damage in the dog or any other animal unless more than 100 mg. per kilogram body weight of cinchophen has been administered.[1, 3, 7] Even then the gastroenteritis is so pronounced that one could

argue that the hepatic damage is secondary to the gastroenteritis. The only exception to the foregoing interpretation was Dog 17 (Table I), in which cholic acid synthesis was depressed definitely during two of three periods of cinchophen administration in the absence of gastrointestinal symptoms. Since diarrhea and vomiting did not occur in this animal, it appears as if the cinchophen was responsible for the depression of cholic acid synthesis. It is worthy of note that in this animal the bile was not being returned to the intestine.

SUMMARY

Seven dogs with bile fistulas with a duodenal fistula for the return of bile to the intestine, which were especially susceptible to the gastrointestinal irritation of cinchophen, were given doses of the drug (approximately 100 mg. per kilogram of body weight) equal to or twice the average human dose. Only seven of sixteen dogs with bile fistulas manifested the symptoms of anorexia, vomiting, or diarrhea under the conditions of our experiment. The output of cholic acid was studied under three conditions: (1) when the drug might affect cholic acid synthesis from a standard diet alone; (2) when the drug might affect both cholic acid synthesis and bile salt secretion as a result of the drug and bile salt excreted in the bile being subjected to three enterohepatic circuits daily; and (3) when the liver was continuously exposed to the cinchophen excreted in the bile by the hourly return of the bile. It was found that any changes in cholic acid synthesis and secretion observed in these susceptible dogs could be accounted for by the gastrointestinal irritation caused by the drug with but one exception. On repeated exposure of the dogs to the drug the gastrointestinal tract became more tolerant, and cholic acid synthesis and secretion were not disturbed.

The observations on the effect of cinchophen on cholic acid synthesis and secretion are consonant with the failure of others to find evidence of microscopic injury of the liver when similar doses of the drug were administered to dogs.[3] However, the one exception referred to shows that the drug may occasionally act on the liver to decrease cholic acid synthesis in the absence of objective symptoms of gastrointestinal irritation.

REFERENCES

1. Lehman, A. J., and P. J. Hanzlik: Cinchophen Toxicosis; Arch. Int. Med. 52: 471, 1933.
   Reichle, H. S.: An Attempt to Produce Toxic Cirrhosis of Liver in Rats Using Cinchophen, Arch. Int. Med. 49: 215, 1932.
2. Rous, P., and McMaster, P. D.: A Method for the Permanent Sterile Drainage of Intra-abdominal Ducts, as Applied to the Common Bile Duct, J. Exper. Med. 37: 11, 1923.
3. Stalker, L. K., Bollman, J. L., and Mann, F. C.: Effect of Cinchophen on the Liver and Other Tissues of the Dog, Proc. Soc. Exper. Biol. & Med. 35: 158, 1936.
4. Reid, P. E., and Ivy, A. C.: *Gastric Mucin, a Prophylactic Against Gastro-Intestinal* Ulcers and "Acute" Toxicity Resulting From Cinchophen, Proc. Soc. Exper. Biol. & Med. 34: 142, 1936.
5. Davis, M. W., Bradley, W. B., Bachrach, W. H., and Ivy, A. C.: Effect of Rammstedt Operation on Incidence of Cinchophen Ulcer, Proc. Soc. Exper. Biol. & Med. 45: 66, 1940.
6. Stalker, L. K., Bollman, J. L., and Mann, F. C.: Effect of Cinchophen on Gastric Secretion, Arch. Surg. 34: 1172, 1937.
   Stalker, L. K., Bollman, J. L., and Mann, F. C.: Experimental Peptic Ulcer Produced by Cinchophen, Arch. Surg. 35: 290, 1937.
7. Barbour, H. G., and Fisk, M. E.: Liver Damage in Dogs and Rats After Repeated Oral Administration of Cinchophen. J. Pharmacol. & Exper. Therap. 48: 341, 1933.

Barbour, H. G., and Gilman, A.: Cinchophen and Tolysin: Comparison of Effects of Administration of Each in Rats. J. Pharmacol. & Exper. Therap. 55: 400, 1935.
Churchill, T. P., and Van Wagoner, F. H.: Cinchophen Poisoning. Proc. Soc. Exper. Biol. & Med. 28: 581, 1930.
Furth, O., and Scholl, R.: Biochemical Studies on Mechanism of Phenylcinchoninic Intoxication, J. Lab. & Clin. Med. 18: 991, 1933.
Knoble, R. M., and Smith, H. A.: The Effect of Cinchophen on Liver of White Rats, Am. J. Physiol. 97: 537, 1931.
Meyers, H. B., and Goodman, L.: Cinchophen Hepatitis, Arch. Int. Med. 49: 946, 1932.
Radwin, L. S., and Lederer, M.: Effect of Cinchophen on the Albino Rat. Arch. Path. 15: 490, 1933.
8. Westfall, G. A.: Toxicity of Cinchophen—Clinical Study, J. Kansas M. Soc. 37: 311, 1936.

RELATION BETWEEN AMOUNTS OF PERTUSSIS ANTIGEN INJECTED AND PRODUCTION OF AGGLUTININS

John A. Toomey, M.D., William S. Takacs, B.S., Roy Averill, M.D., and Norman Lewis, M.D., Cleveland, Ohio

I

The freshly isolated disease-producing *Hemophilus pertussis* organisms called by us phase A[1, 2] and phase I by Leslie and Gardner[3] are used in active immunization in man. Although phase B organisms, termed by us the "nondisease-producing" stage (the "resting stage," the "fixed" bacilli, and phase IV by Leslie and Gardner), descend from the phase A types, they are said to be worthless as immunization agents. Guinea pigs can be protected against lethal doses of phase B organisms by the previous injection of phase B vaccine, but no protection is established against phase A organisms by actively immunizing these animals with phase A vaccines. Doses of the standardized phase B antigen (4 mg. of wet weighed organisms to 1 c.c.) used in previous experiments failed to produce any agglutinins against phase A bacilli.

The relative size of organisms in the two phase conditions is significant, the phase B type being 50 or even more times the size of phase A. One milligram of wet weighed phase A organisms contains definitely more bacteria than an equal amount of wet weighed phase B bacilli.

Years ago, Huenekens[4] stated that pertussis vaccines (phase B state) should be given in massive doses to secure results. Sauer[5] has recommended even larger doses of freshly isolated *H. pertussis* organisms (phase A state) for active immunization.

The size of the organism may conceivably be related to the ability to immunize and produce antibodies. Were the latter true, perhaps increased amounts of phase B organisms used as the stimulating antigen and injected into rabbits would produce a higher agglutinin titer in their blood serums against homologous and heterologous organisms than would smaller amounts.

From the Division of Contagious Diseases, City Hospital, and the Department of Pediatrics, Western Reserve University, Cleveland.
Received for publication, Aug. 31, 1942.

Methods and Materials.—No. 778 stock phase B strain culture (American Type Culture Collection) was grown in veal brain liquid medium.[6a] The viscid, sticky mass produced by the organisms was centrifuged down as much as possible, the supernate discarded, and the centrifugate dried and powdered in a lyophile apparatus. Some of the toxin previously described was nearly always present in this specimen.[6b] Twenty milligrams of the powder were added to each 1 c.c. of normal saline. Part of this 20 mg. suspension was diluted to make a standardized suspension of 4 mg. in 1 c.c. of normal saline. Eight rabbits were injected, four with the dose standardized at 4 mg. and four at 20 mg. The injections were given intravenously the same day and in the same amounts as previously described.[1]

The animals were bled ten days after the last injection of antigen. The blood was put in the refrigerator overnight, and the next day the blood serum was separated off by centrifugation. Agglutination tests were then done with twelve strains of *H. pertussis* (phase B state) that had become acclimated to veal brain agar. The type of agglutinin response has been described previously.[1]

There was an obvious increase in agglutinin titer in the blood serums of the four animals that received the larger doses of phase B organisms in toxin. The titer of agglutinins present in these latter animals was not tested to its end point in all instances, although with the smaller dosages an end zone was obtained.

The phase B antigen-antibody serum agglutinated Walker strain 4 plus at 1:640 and had no reaction at 1:1280. The exact end point between 1:640 and 1:1280 was not determined, but it was not pertinent since our objective was simply to determine if larger amounts of the same antigen, in this instance phase B organisms, in a small amount of toxin would cause an increase in agglutinin titer. The blood serum agglutinin titers of strains Walker, Nos. 13 and 15, were at least twice as high as that obtained with the lesser amount of antigen; those of Betty, Brescan, Nos. 5, 8, 12, 28, and 778, were at least four times as high; that of No. 23, at least eight times as high; and that of No. 9, sixteen times as high.

The results were clear-cut, and it could be concluded that when five times the volume of *H. pertussis* phase B organisms "in toxin" was used as an antigen, a relatively higher titer of agglutinating antibodies was produced than with one-fifth the volume of phase B organisms "in toxin."

The phase B serums were also tried with phase A organisms. The rabbit serums obtained by injecting 4 mg. of the antigen were consistently found wanting in agglutinin titer, as was found in a previous experiment where similar standardized doses of organisms were used as the antigen. The rabbit serum obtained by injecting 20 mg. of antigen showed suggestive plus-minus agglutination reactions in the 1:40 titer.

This might suggest the presence of specific agglutinins. However, although the phase A organisms used had all the qualities and characteristics of a phase A bacillus and had been carried on human blood potato agar, definite conclusions could not be reached, since all but one of these phase A organisms had been isolated several months before the experiment was done

and some of their phase A quality might have been converted into phase B quality, thus changing their reacting ability to specific serum. Especially was this suggestive since the one phase A organism isolated but twenty-six days prior to the experiment was not agglutinated in any dilution by phase B, 4 mg. or 20 mg. serums. Although phase B 20 mg. serums had been found to possess higher agglutinin titers than did phase B 4 mg. serums, it was still not known whether more antigen could produce a higher titer in the absence of what might be an enhancing "toxin."

This experiment with the dried antigen and toxin was repeated; the serums obtained were agglutinated against eight freshly isolated organisms that had been carried in their phase A state and the same organisms which had been acclimated to and grown on veal brain agar to a phase B state. The results were similar.

II

In the previous experiment, phase B organism antigen contained a "toxin" not present in the ordinary commercial vaccines. It was decided in so far as possible to work with pure organism antigens.

A relatively small amount of standardized phase A and B organisms had been used to inject the rabbit to produce agglutinins in the blood serums.

If the agglutinin titer of the serum were dependent upon the number of organisms injected, and if this number were increased more than in the last experiment and the "toxin" factor excluded, with the exception of that contained in the water of condensation, it might be that there would not only be an increase in blood serum agglutinin titer against the homologous phase B, but also against the heterologous phase A organisms.

Methods and Materials.—In the previous experiments, the organisms were grown in liquid media and "toxin" was produced. In this experiment, No. 778 *H. pertussis* stock phase B strain was grown on solid media brain veal agar for two days and mass production of "toxin" avoided. The growth was scraped off, wet weighed, and standardized at 4 mg. per cubic centimeter, 20 mg. per cubic centimeter, and 45 mg. per cubic centimeter. Two-tenths per cent formalin was added to the various standardized organism suspensions. The latter were shaken every day and kept in the refrigerator for seven days. Several rabbits weighing between 1 and 1½ pounds were bled and their serums tested for the presence of normal agglutinins, since occasionally agglutinins against *H. pertussis* are found in normal rabbits. When six animals had been obtained, the blood serums of which lacked normal agglutinins against either phase A or B organisms, two each were injected intravenously every four days with a different one of the three standardized suspensions, the dosages being 0.2 c.c., 0.5 c.c., 0.8 c.c., 1.2 c.c., 1.5 c.c., 1.8 c.c., 2.0 c.c., and 2.3 c.c. The rabbits were bled ten days after the last injection. The blood was left in the refrigerator overnight to allow clotting and was centrifuged the next morning until the serum was clear; it was then inactivated one hour at 56° C., and merthiolate preservative was added. Agglutination tests were done against homologous phase B and heterologous phase A organisms. There were three bleedings, on the thirty-second, forty-sixth, and sixtieth days after the last injection.

It was easy to inject rabbits intravenously with 4 mg. per cubic centimeter and 20 mg. per cubic centimeter of a standardized suspension of organisms, but difficulties were encountered with the 45 mg. per cubic centimeter dose. One of the rabbits (No. 149) injected with the latter amount died after the second dose of the antigen. One of the two animals substituted died after the first injection. Several other normal rabbits lacking agglutinins against phase A or phase B *H. pertussis* organisms were given the 45 mg. per cubic centimeter dose, so that even though the mortality rate might be high during the process of immunization, enough animals would remain alive at the end of the procedure to finish the experiment. Three of eight animals injected with the 45 mg. per cubic centimeter dose died during the course of injections.

Agglutination tests were set up against (1) twelve phase A organisms isolated and grown on human blood agar within a few months before the experiment was started and (2) six phase B organisms isolated several years prior and carried on veal brain agar. The first bleeding was done thirty-two days after the last antigen injection against many phase A organisms. There was an increase in agglutinins in the blood serums of three rabbits (Nos. 190, 120, and 147) injected with 45 mg. per cubic centimeter, and against a few phase A organisms in the serums of two others (Nos. 21 and 180). This ability to agglutinate had practically disappeared in the specimens of the second and third bleedings obtained forty-six and sixty-six days later. Thus, agglutinins against some phase A organisms can be produced by phase B bacilli, although to a titer of only 1:80. These agglutinins tend to disappear within a month or so after the last antigen injection.

These rabbits injected with 4 mg. per cubic centimeter or 45 mg. per cubic centimeter of phase B antigen showed good titer responses against their homologous phase B organisms in the first blood serums obtained. There might have been a slight increase in titer value with the 45 mg. per cubic centimeter antigen, but it was not too definite. There was an increase in the agglutinin titers against phase B organisms in the specimens of the second bleeding, which was about equal in all serums regardless of the amount of antigen stimulus, whether 4 mg. per cubic centimeter or 45 mg. per cubic centimeter. The agglutinin titers in the specimens of the third bleeding had decreased to approximately those of the first blood serums and were about the same no matter what the stimulus.

It could be concluded that there was an increase in agglutinins against phase A organisms found in the first blood serums of the animals injected with 45 mg. per cubic centimeter of phase B, a titer lost only sixteen days later; and there was a good and almost equal response to all phase B organisms, irrespective of the stimulating phase B antigen and the time of bleeding, the highest titer being found in the blood specimen withdrawn forty-six days after injection. This conclusion differs from that of Experiment I, but the previous results could be easily explained because of the differences in the stimulating antigen, since only organisms were used in this experiment, and in the former work the organisms were contained in toxin, which possibly enhanced their antigen effect.

III

Stimulation with more antigen made from phase B organisms did not seem to increase the titer production against phase B organisms. However, there did seem to be a slight increase against the heterologous phase A organisms, so that it was thought that the surviving animals of Experiment II should be stimulated further.

All five animals of Experiment II that had survived the dose of 45 mg. per cubic centimeter of the phase B antigen were injected weekly for ten weeks with 2 c.c. of phase B organisms standardized at 45 mg. per cubic centimeter. There was an eleven-day interval between the first injection in this series and the final bleeding of the last series. Two of the five animals died; each of the remaining three were bled three times—twelve, twenty-three, and forty-four days after the last injection.

Agglutination tests were done with the nine blood serums of the three animals against five phase A organisms (Nos. 1, 2, 6, 7, and 8) isolated several months previously, five phase A organisms (Nos. 3, 4, 5, 9, and 10) isolated within the previous month, and three phase B organisms. The titers of the blood serum against phase B organisms were as usual, while against some heterologous phase A organisms, they were at least 1:80.

In a collateral experiment, agglutination tests were done against three phase B organisms and ten organisms which had been carried on human blood agar for several years, organisms which were beginning to show some phase B qualities. The latter, though carried on blood agar, were agglutinated by rabbit blood serums, in most instances to a titer of at least 1:320, thus demonstrating some approach to a phase B state.

IV

It was difficult to secure enough freshly isolated organisms to start a simultaneous experiment with many organisms where all the experimental factors would be the same. Some organisms grow faster than others and, as in the last experiment, a month or more might elapse before an experiment can be started. Meanwhile, the organisms, although grown on blood, might be progressing to a phase B state so that the titer obtained by phase B organism antibody serum might represent a reaction against some B-ness which was beginning to appear with age in the phase A organisms. To settle this point, another set of experiments were done. This time organisms were isolated, grown a few times on human blood agar, frozen in a lyophile apparatus, and set aside until at least five strains had been collected. By this method, the phase A characteristics were neither altered nor lost, and the organisms remained in their originally isolated state. The method of procedure was the same as in Experiment III.

Here again it was demonstrated that the homologous phase B organisms can produce some agglutinins against the heterologous phase A bacilli. These reactions are noted in Chart 1. Of the three rabbits used, the agglutinin titers of the blood serums of Nos. 147 and 190 were the highest after the first bleeding, and those of rabbit No. 21 might have been considered the same or slightly higher after the second bleeding. No animal had much of an increase in phase A agglutinins after the third bleeding.

COMMENT

We still use the terms phase A and phase B and not phases I, II, III, and IV as do Leslie and Gardner.[3] This is not in agreement with Dozois, Flosdorf, and Kimball.[7] The latter, in their work, constructed certain artificial boundaries and attempted to demonstrate three phases of existence. Yet at the same time they presented data concerning certain strains in various of their phases, which, because they did not fall within the limits of their phase boundaries, were described as "unrepresentative" and atypical"—and this despite the fact that these very exceptions pointed to the existence of an imperceptibly graded transition between two extremes of statistical maxima and minima; namely,

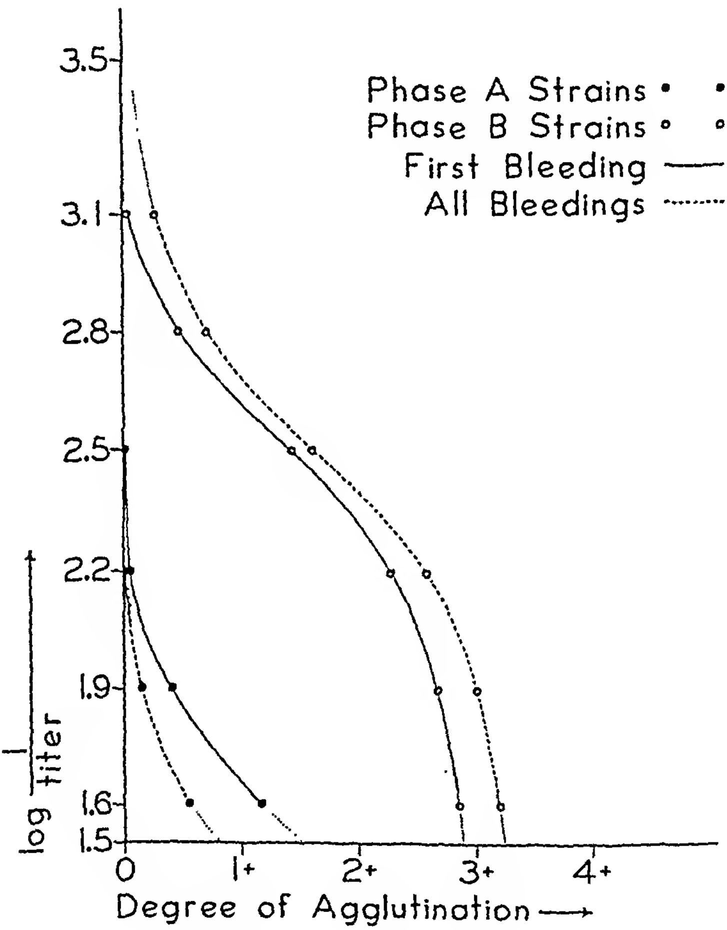


Chart 1.—Illustrating results of Experiment IV.

phases A and B (phase A, disease-producing stage; phase B, resting or non-disease-producing stage). Indeed, they explained away their "unrepresentative" strains by concluding that the phase relationships of *H. pertussis* are "more complex than was found by Leslie and Gardner," a conclusion with which we fully agree. In fact, the relationships are so complex that it would seem impractical to consider any other phases than the two previously mentioned.

Flosdorf and associates leave unexplained other data at variance with their theories, chiefly concerning the relationships between the cross-agglutination and cross-absorption characteristics of their phases I and III. They dismiss the fact "that the whole phase I organisms absorb phase III agglutinins" as "irrelevant, quantitatively, because other factors may be concerned in such reactions with particulate matter," a statement, which if true, should indicate that any agglutination or absorption test is subject to the same errors and is of proportionate relevance.

Flosdorf and associates did not agree with the premises on which we disputed Leslie and Gardner's assumptions as to phase existence. It has been shown, however, that *H. pertussis* organisms, if grown long enough on artificial media, with or without blood, gradually change their characteristics and in time reach a state in which all subcultured organisms, no matter what their original characteristics, acquire the ability to produce about the same degree of agglutinin titer in the blood serums of rabbits, have the same virulence, and appear morphologically the same as other phase B organisms carried on hemoglobin-free media. At the other extreme are the organisms freshly isolated from the human being ill with the disease, organisms which at the moment of isolation approach more or less the ideal opposite of the fixed stage. However, *H. pertussis* organisms seldom, if ever, attain either ideal extreme, for it has been shown here that by sufficiently intense programs of immunization it is actually possible to obtain cross-agglutinations between phases A and B. Would it not be theoretically possible to isolate any organism anywhere in its transition toward the resting stage at a time when it is losing some of the characteristics of phase I and acquiring some of the characteristics of phase IV and to immobolize it in this stage of its existence by a process of freezing and drying? This should not be impossible, since these authors themselves have demonstrated the existence of certainly more than four stages (phase variations).

It is our belief that the so-called phase II and phase III are only a few of the many possible "immobilizations of a phase" which might appear in the transition of organisms toward the resting or phase B state. The only two phases of which we ourselves are confident are (1) phase A, the disease-producing stage which in its freshly isolated state may have already started somewhat on its way toward the resting state and (2) phase B, the resting state.

Inferentially it could be said that if agglutinin production were a sina qua non of a good immunizing antigen, phase B organisms should be considered deficient as vaccines for human use.

SUMMARY

1. Rabbits given ten doses of phase B *H. pertussis* organisms standardized in toxin at 20 mg. per cubic centimeter have a higher agglutinin titer against phase B organisms than do the blood serums of those given the same number of doses of 4 mg. per cubic centimeter. Both antigens produced only a negligible titer of agglutinins against phase A organisms, the type which initiates the disease in man.

2. Four milligrams, 20 mg., and 45 mg. of phase B organims for ten injections will produce nearly comparable agglutinin titers in rabbits against phase B organisms and some demonstrable but not marked agglutinins against heterologous phase A bacilli.

3. When rabbits are injected weekly over a long period with massive amounts of phase B organisms as the antigen, there is no increase in titer against the phase B organisms, but in some instances there may be an increase in the agglutinin titer against phase A bacilli—as high as 1:160.

REFERENCES

1. Toomey, J. A., Ranta, K., Robey, L., and McClelland, J. E.: The Phases or Types of H. pertussis, J. Infect. Dis. 57: 49, 1935.
2. Toomey, J. A., Ranta, K., and Takacs, W. S.: The Virulence of H. pertussis, J. Infect. Dis. 57: 286, 1935.
3. Leslie, P. H., and Gardner, A. D.: Phases of Haemophilus pertussis, J. Hyg. 31: 423, 1931.
4. Huenekens, E. J.: The Prophylactic Use of Pertussis Vaccine Controlled by the Complement-Fixation Test, Am. J. Dis. Child. 14: 283, 1917.
5. Sauer, L. W.: Whooping Cough: Resume of Seven Years' Study, J. Pediat. 2: 740, 1933.
6. a. Toomey, J. A., and McClelland, J. E., Pertussis Toxin or Antigen, Proc. Soc. Exper. Biol. & Med. 31: 34, 1933.
   b. Toomey, J. A., McClelland, J. E., and Lieder, L. E.: Further Experiments With B. pertussis Filtrate, Proc. Soc. Exper. Biol. & Med. 31: 403, 1933.
7. Flosdorf, E. W., Dozois, T. F., and Kimball, A. C.: Studies With H. pertussis, V. Agglutinogenic Relationships of the phases, J. Bact. 41: 457, 1941.

AN EXPERIMENTAL STUDY OF PHLEBITIS FOLLOWING VENOCLYSIS WITH GLUCOSE AND AMINO ACID SOLUTIONS

Abraham Horvitz, M.D., L. A. Sachar, M.D., and Robert Elman, M.D.,
St. Louis, Mo.

Although injury to veins during intravenous injections is of frequent clinical occurrence, often producing thrombosis sufficient to preclude further use of the vessel, there have been few experimental studies of the various factors which influence its degree and severity. Presumably these influencing factors are numerous, some of which are as follows: The character of the solution (its chemical properties, its pH, and its osmotic pressure), the size of the vein injected, the rate and duration of injection, the trauma produced in introducing the needle, the trauma produced by the needle while resting in the vein, stasis resulting from the partial occlusion of the vein by the needle, and the previous state of health of the vein.

The present study was undertaken primarily to determine whether the presence of amino acids, especially in solutions of glucose, influences the degree of injury produced by intravenous injections as compared with simple solu-

From the Department of Surgery, Washington University School of Medicine and Barnes Hospital, St. Louis.

Received for publication, Sept. 3, 1942.

tions of glucose. As far as possible, all factors other than the character of the solution were kept constant.

METHOD

Dogs weighing approximately 10 kilograms were the experimental animals. The injections were carried out under nembutal anesthesia. Two pairs of veins were used: i.e., the large vein on the dorsum of both forelegs, known as the antebrachial cephalic, and the somewhat smaller, saphenous vein coursing along the inner aspect of both hind legs. The solution to be tested was administered on one side, the control solution simultaneously on the other side. Twenty-four hours later the two remaining veins were similarly injected. After a second twenty-four-hour period the dog was killed and the veins removed for section and study. Thus in most experiments each dog furnished two sets of veins, one showing changes twenty-four hours and one forty-eight hours after injection. The amount and rate of fluid injected were determined by the weight of the dog. Six cubic centimeters per kilogram per hour were accepted as fairly closely approximating the amounts used clinically. Because a three-hour injection usually produced definite changes with the first solutions used, this period was adopted. Twenty-gauge needles were used in all experiments, except in a few, which will be referred to below, when a twenty-five-gauge needle was used. The solutions were introduced at room temperature. The position of the point of the needle in the vein was marked by a silk suture placed in the skin just above it so that microscopic comparison could be made at the same distance from the point of injection. In early experiments sections were made longitudinally through the middle of the vein. In later experiments, cross-sections were taken two to three millimeters apart for use. The sections were stained with hematoxylin and eosin. In the study of many sections it was found that any injury observed was always greatest near the point where the end of the needle had rested. Thus, a section of vein taken about 1 cm. to either side of the needle point was used for the comparison.

Evaluation of Changes in the Veins.—In recording the changes in the veins, all observations were divided into those noted in the intima, those in the media, those in the adventitia, and also those within the lumen of the vessel. One of the earliest intimal changes observed in these sections was a vacuolation of the endothelial cells. This change might be observed in the absence of any other changes in the vessel wall. With further injury there occurred progressive cellular changes with ultimate death of the cells and destruction of the endothelial lining. Often, though not always, the denuded intima furnished a locus for the deposition of fibrin, and varying degrees of thrombus formation were observed. In the media the observed changes ranged from slight pyknosis of some of the nuclei to complete necrosis of the muscular wall. To establish a basis for comparing and evaluating the changes, they were classified as follows: (1) changes within the lumen, i.e., thrombosis: (2) changes in the intima, i.e., (a) endothelial vacuolation, (b) endothelial destruction: (3) changes in the media, i.e., (a) edema, (b) leucocytic infiltration, (c) hemorrhage, (d) necrotic changes; and (4) changes in the adventitia, i.e., (a) edema, (b) leucocytic infiltration, (c) hemorrhage. This classification is similar to though less detailed

TABLE I

EVALUATION OF THE SCORE INDICATING DEGREE OF DAMAGE TO VEIN DURING VENOCLYSIS

VEIN	SOLUTION	LUMEN	INTIMA		MEDIA			ADVENTITIA				HOURS	TOTAL SCORE
		THROMBOSIS	ENDOTHELIAL VACUOLIZATION	ENDOTHELIAL DESTRUCTION	EDEMA	HEMORRHAGE	LEUCOCYTIC INFILTRATION	NECROTIC CHANGES	EDEMA	HEMORRHAGE	LEUCOCYTIC INFILTRATION		
Right foreleg	11.4% G.	0	3	0	2	0	0	0	2	0	0	48	7
Left foreleg	5.0% A. 5.0% G.	2	3	2	2	0	2	2	3	0	0	48	16
Right hindleg	11.4% G.	2	3	2	1	0	2	2	3	0	2	24	17
Left hindleg	5.0% A. 5.0% G.	3	3	3	1	2	2	3	2	0	2	24	21

G., Glucose.
A., Amigen (hydrolyzed casein).

than that used by Ochsner and Garside[1] and by Ochsner and Mahorner[2] in their experiments on the effects of various sclerosing solutions used in the treatment of varicose veins. Each of the foregoing changes was recorded according to its severity. The absence of any change was rated as zero. Slight or early changes were rated as one, moderate changes as two, and advanced changes as three. By adding the ratings for each vein the final estimate or score of the severity of the phlebitis took the form of a total which was a rough numerical estimate of the degree of injury inflicted by the solution. While this type of scoring was based on the subjective evaluation of the degree of change seen, the observations were objective in so far as they were made with no knowledge as to which solution had been injected into the vein. When a number of the sections were rated separately by a second observer, the total scores obtained agreed rather well. A typical example of the method of scoring used is shown in Table I.

The amino acid solution tested in all experiments was made from an enzymatic hydrolysate of casein (Amigen*). The pH of this product in solution is approximately 4.5; when a more alkaline solution was tried, sodium hydroxide was added until the desired pH was reached. A 2.7 to 3.0 per cent concentration of the product in solution has been found to be isotonic with the blood; this is slightly greater than glucose. In comparing two solutions, each was isotonic.

FINDINGS

Eleven dogs were obtained for study, of which seven yielded four veins each, four but two veins each. In all, eighteen pairs of veins were observed. A summary of the scores is listed in Table II. The results are also described in detail as follows:

1. A 2½ per cent amigen solution in 10 per cent glucose was one of the first solutions tested against a control of 13 per cent glucose which has an osmotic pressure approximately equal to that of the amigen solution, as measured by its freezing point. In all three experiments (1, 1a, 2) no significant difference was found between the injury produced by the tested and the control solutions, although definite injury was produced in each case. The tendency to thrombus formation was frequent. When the injection of both test and control solutions was repeated twice in the same veins, thrombus formation and damage to the entire vein was marked. In all experiments injury produced by the same solutions was greater in the veins of the hind legs than in those of the forelegs. This was probably due to stasis because of the smaller caliber of the former; this factor is discussed below.

2. A solution containing 5 per cent amigen and 5 per cent glucose was tested against an 11.4 per cent glucose solution, the latter having an osmotic pressure equal to the former (Experiments 3 and 3a). In the forelegs the injury produced by the amino acid solution was greater than that caused by the glucose. In the veins of the hindlegs the damage produced by both solutions was severe, thrombosis being present in each case, and there was no significant difference between the two.

*Prepared by Mead Johnson & Co.

TABLE II

SUMMARY OF SCORES OBTAINED WITH VARIOUS SOLUTIONS

EXPERIMENT	VEIN	SOLUTION	HOURS	SCORE
1	R. F.	13% G.	24	12
	L. F.	2½% A., 10% G.	24	11
1a	R. H.	13% G.	48	20
	L. H.	2½% A., 10% G.	48	22
2	R. F.	2½% A., 10% G.	24	13
	L. F.	13% G.	24	19
3	R. F.	11.4% G.	48	7
	L. F.	5.0% A., 5% G.	48	16
3a	R. H.	11.4% G.	24	17
	L. H.	5.0% A., 5% G.	24	21
4	R. F.	5% A., 5% G.	24	5
	L. F.	8.8% A.	24	13
4a	R. H.	8.8% A.	48	9
	L. H.	5% A., 5% G.	48	14
5	R. F.	7.7% A.	24	18
	L. F.	10% G.	24	4
6	R. F.	7.7% A.	24	13
	L. F.	10% G.	24	15
6a	R. H.	7.7% A.	48	22
	L. H.	10% G.	48	18
7	R. F.	7.5% A., pH-7.3	48	4
	L. F.	7.5% A., pH-4.6	48	8
7a	R. H.	7.5% A., pH-4.6	24	20
	L. H.	7.5% A., pH-7.3	24	9
8	R. F.	7.5% A., pH-7.4	48	6
	L. F.	7.5% A., pH-4.6	48	13
9	R. F.	7.5% A., pH-4.6	24	20
	L. F.	7.5% A., pH-7.4	24	7

R. F., Right foreleg.
R. H., Right hindleg.
L. F., Left foreleg.
L. H., Left hindleg.
G., Glucose.
A., Amigen.

3. A solution containing 5 per cent amigen and 5 per cent glucose was tested against an isotonic amigen solution (8.8 per cent) and the score was even, i.e., in one experiment (Experiment 4) the former solution produced less damage, whereas in the other experiment (Experiment 4a) it produced more.

4. A 7.7 per cent amino acid solution, isotonic with a 10 per cent glucose solution, was tested against the latter (Experiments 5, 6, 6a). Thrombus formation was present in each case and the damage done to the vein wall was essentially the same for the two solutions in Experiments 6 and 6a, whereas in Experiment 5 the score for the amigen solution was eighteen against four for the glucose.

5. The significance of the pH of the amino acid solutions as a factor was also tested (Experiments 7, 7a, 8, and 9). In all experiments a 7.5 per cent solution neutralized to a pH of 7.4 produced less damage than the one left at pH 4.6; the difference was striking in three of the four experiments.

6. Early in these experiments, as noted above, it seemed trauma and stasis played an important part in the production of thrombosis. A small gauge needle, No. 25, was therefore tested against the larger standard 20-gauge needle. The same solution was, of course, injected through each needle at the same rate.

In one set of experiments (Experiments 10 and 10a) 2½ per cent amino acid in 10 per cent glucose was used. Here injury was greater in the veins injected with the larger (No. 20) gauge needle, this causing thrombus formation, whereas none was produced by the smaller (No. 25) needle. In the second pair of experiments (Experiments 11 and 11a) a 13 per cent glucose solution was used. No significant difference was found between the two needles. The different results obtained in these two sets of experiments may be due to a difference in the caliber of the veins of the dogs used.

COMMENT

Careful perusal of the scores listed in Tables II and III indicated the greater damage inflicted by the more hypertonic solutions, confirming many clinical observations. On the other hand, solutions containing 10 per cent glucose or less or its equivalent tend to produce less phlebitis. The presence of amino acids seems to have but slight effect on the amount of damage produced, although it is clear that the pH is an important factor. This probably explains the difference noted in Experiment 5, the only one in which there was a definitely increased phlebitis with amigen alone as compared with glucose alone. This effect is clearly demonstrated in Experiments 7, 7a, 8 and 9, in which the neutralized amigen provoked much less phlebitis than the unneutralized solution in all four sets of veins.

TABLE III

SUMMARY OF SCORES ATTAINED WITH DIFFERENT NEEDLES

EXPERIMENT	VEIN	NEEDLE	HOURS	SCORE	SOLUTION
10	R. F.	25	48	3	2½% Amigen
	L. F.	20	48	6	10% Glucose
10a	R. H.	20	24	17	2½% Amigen
	L. H.	25	24	4	10% Glucose
11	R. F.	20	24	13	13% Glucose
	L. F.	25	24	17	
11a	R. H.	20	48	26	13% Glucose
	L. H.	25	48	24	

The role of stasis and obstruction deserves some comment as already indicated. While a smaller needle should allow blood to flow around it, thus preventing stasis, this depends on the relative caliber of the lumen as compared with the gauge of the needle. It was our definite impression that this accounted for variations between the hind- and forelegs, although the objective evidence herein adduced does not permit such a conclusion. The considerations are of practical importance and deserve further study.

CONCLUSIONS

1. A method of scoring is proposed to measure the degree of phlebitis produced by intravenous injections.

2. Phlebitis is pronounced following a three-hour intravenous injection of a 2½ per cent amino acid-10 per cent glucose solution, due not to the presence of the amino acids but to the hypertonicity of the solution, inasmuch as the same changes are produced by isotonic (13 per cent) glucose solutions. Less

pronounced changes occur with more hypotonic solutions containing either amino acids or glucose or both.

3. Acid solutions tend to produce more phlebitis than neutral solutions as shown by comparing the damage produced by a 7.5 per cent solution of amigen at a pH of 4.6 with the same solution neutralized to pH 7.4.

4. The size of the needle used probably plays an important part in thrombus formation, although this factor is undoubtedly associated with the relative caliber of the veins.

REFERENCES

1. Ochsner, A., and Garside, E.: The Intravenous Injection of Sclerosing Substance, Ann. Surg. 96: 691, 1932.
2. Ochsner, A., and Mahorner, H.: Comparative Value of Intravenous Sclerosing Substance, Arch. Surg. 29: 397, 1934.

ERYTHROCYTE LONGEVITY IN DOGS AND RABBITS*

JOHN EMERSON DAVIS, M.S., PH.D.
LITTLE ROCK, ARK.

NUMEROUS estimates have been made of the life duration of red blood corpuscles in various species. Ashby,[1] on the basis of agglutination experiments, has placed the life span of human transfused erythrocytes at thirty days or more. Eaton and Damren[2, 3] have studied the reticulocyte percentage peaks in rabbit blood following hemorrhage and have concluded that the life span of red cells in this species is about 8.5 days. Escobar and Baldwin[4] exposed animals to reduced atmospheric pressures to increase the number of red cells and then observed the time required for their return to normal at atmospheric pressure. This period of time was considered by the authors to represent the life duration of erythrocytes in the circulation. They thus obtained values of twelve to eighteen days in rats, sixteen to twenty-three days in dogs, and eighteen to thirty days in man. Hawkins and Whipple,[5] from a study of bile pigment output in dogs with biliary fistulas following hemorrhage or acetylphenylhydrazine, have reported that the life span of erythrocytes in this species is about 124 days.

The present communication presents a new approach to this subject through the depression of erythropoiesis by drugs in polycythemic animals. The experimental data for my calculations of erythrocyte longevity have been taken from previous papers[6-9] and other data of mine.

The method involves the production of experimental polycythemia in dogs or rabbits by daily exercise,[6] continued administration of cobalt chloride,[6-9] ex-

---

*Research paper No. 532, Journal series, University of Arkansas.
From the Department of Physiology and Pharmacology, University of Arkansas School of Medicine, Little Rock.
Received for publication, Sept. 9, 1942.

posure to low atmospheric pressure[7] or daily ephedrine administration.[9] The high erythrocyte counts were then reduced by the additional daily administration of raw liver, choline hydrochloride, mecholyl, or other vasodilator drugs. It has been found that only a few days are required for these substances to reduce the red cell counts to or toward normal to points which seem to represent the maximal depression that these drugs are capable of producing. It is assumed that these drugs act as a "brake" on erythrocyte production until the cell count reaches normal, or near normal. If such is the case, the rate of depression of the polycythemia should establish the rate of natural death of the erythrocytes, and it is then possible to calculate the length of time which would be required for 100 per cent disappearance of the cells if the process were to continue. This calculation has been made from composite or average curves drawn from rabbit and dog experiments.

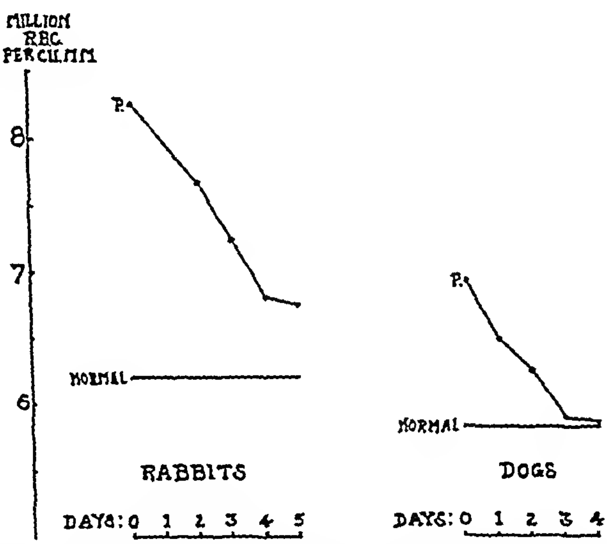


Fig. 1.—Average curves for rabbit and dog erythrocytes, showing the depression of experimental polycythemia by liver and various vasodilator drugs. *P* designates the average polycythemic level for each species.

RESULTS

Fig. 1 shows curves representing the depression by drugs of the average erythrocyte counts from polycythemic values to near normal in rabbits and dogs.

The average curve for rabbits is composed of twelve experiments on seven rabbits. It will be seen that four days were required for a practically maximal reduction of 17.8 per cent in the polycythemic cell count to occur. If this rate of reduction were to continue, all erythrocytes would disappear in 22.4 days.

The curve for dog erythrocytes represents thirty-three experiments on twenty dogs. It shows that a practically maximal reduction of 15 per cent of the polycythemic cell count occurred in three days. At this rate the last of the dog corpuscles would disappear in twenty days.

On the basis of these calculations, the minimal length of life of erythrocytes should be placed at twenty days in the dog and at about twenty-two days in the rabbit.

DISCUSSION

Our evidence for believing that the reductions of erythrocyte counts shown in Fig. 1 are due to a depression of erythropoiesis has been previously presented in articles cited above.[6-9] Since the depression occurs in spite of continuation of hemopoietic stimulating measures, and since the red cell count rises again to polycythemic values soon after cessation of administration of the depressant drugs, it seems justifiable to assume that the latter agents act as a "brake" on erythropoiesis. In some manner this brake seems to operate only until the erythrocyte number is reduced to a certain level which is not below the normal. We assume that the bone marrow still has an "excess momentum," so that when the "drug brake" is removed, an accelerated rate of hemopoiesis is able to cause a return of polycythemia in a few days.

Although it is recognized that the assumption that the rate of depression of polycythemia establishes the rate of natural death of erythrocytes is a broad one, it is perhaps permissible to say that it establishes the maximal rate of cell disappearance. Our calculated values for the life span of the erythrocyte should then be considered as minimum values.

CONCLUSIONS

On the basis of data on the rate of depression of experimental polycythemias by liver or vasodilator drugs, the *minimal average life duration* of erythrocytes has been calculated. The "life spans," thus obtained, are twenty days in the dog, and 22.4 days in the rabbit.

REFERENCES

1. Ashby, W.: The Determination of the Length of Life of Transfused Blood Corpuscles in Man, J. Exper. Med. 29: 267, 1919.
2. Eaton, P., and Damren, F. L.: A Method for Determining the Life Duration of the Erythrocyte, South. M. J. 23: 311, 1930.
3. Eaton, P., and Damren, F. L.: A Method for Determining the Life Duration of the Erythrocyte: II. The Rate of Red Cell Production After Hemorrhage, South. M. J. 23: 395, 1930.
4. Escobar, R. A., and Baldwin, F. M.: The Longevity of the Erythrocyte, Am. J. Physiol. 107: 249, 1934.
5. Hawkins, W. B., and Whipple, G. H.: The Life Cycle of the Red Blood Cell in the Dog, Am. J. Physiol. 122: 418, 1938.
6. Davis, J. E.: The Reduction of Experimental Polycythemias by Liver Administration, Am. J. Physiol. 122: 397, 1938.
7. Davis, J. E.: Depression of Experimental Polycythemias by Choline Hydrochloride or Liver Administration, Am. J. Physiol. 127: 322, 1939.
8. Davis, J. E.: The Mechanism of the Depressant Action of Liver and Choline Hydrochloride Upon Experimental Polycythemia. The Effect of Sodium Nitrite and Choline Derivatives on Polycythemic Dogs, J. Pharmacol. & Exper. Therap. 70: 408, 1940.
9. Davis, J. E.: The Depression of Experimental Polycythemia by Various Substances in Dogs, Rabbits, and Man, J. Pharmacol. & Exper. Therap. 73: 162, 1941.

THE EFFECT OF CHANGE OF ALTITUDE ON THE BASAL METABOLISM OF HUMAN SUBJECTS*

ROBERT C. LEWIS, PH.D., ALBERTA ILIFF, PH.D., ANNA MARIE DUVAL, PH.D., DENVER, COLO., AND GLADYS M. KINSMAN,† PH.D., STILLWATER, OKLA.

IN A study of the basal metabolism of the children of the Child Research Council at Denver, sufficient data have been accumulated to establish normal values for different age groups from the third through the sixteenth year. That portion of the data obtained prior to April, 1936, for children between the ages of 2 and 13 years has been reported by Lewis, Kinsman, and Iliff.[1] Since the publication of this study, pediatricians and investigators in the field of energy metabolism have asked from time to time whether the normal values of basal metabolism thus established at an altitude of 5,280 feet can be used as standards at lower altitudes. In discussing this problem from the standpoint of the literature on the subject, the conclusion was drawn[1] that between barometric pressures of 610 and 645 mm. of mercury, at which the children were studied, the partial pressure of oxygen was not sufficiently low to influence their basal metabolism. However, in view of the queries raised as to the dependability of the standards of the Child Research Council at other altitudes, further consideration should be given to the question of whether the altitude at which determinations of basal metabolism are made affects the results.

Numerous investigators have studied the effect of altitude on the energy metabolism of human subjects. Observations[2-19] have been made by determining the oxygen consumption of the same individuals first at or near sea level and then at one or more higher elevations. It may be seen from Table I, which gives the percentage changes at different ranges of altitude from the heat production found near sea level, that considerable variation has occurred. However, no marked effect is evident in the case of altitudes below 10,000 feet. The greatest disagreement appears at the higher altitudes and is particularly noticeable in studies made prior to 1913 when the experimental conditions were not always well controlled. Since that time practically all of the observations have been made under basal conditions, and it may be noted that for altitudes between 10,000 and 15,000 feet the deviation from the results obtained at sea level has been considerably less than in the earlier years.

*From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver, and the Oklahoma Agricultural Experiment Station, Stillwater.

This report is taken from the dissertation submitted by Alberta Iliff to the Faculty of the Graduate School of the University of Colorado, June, 1942, in partial fulfillment of the requirements for the degree Doctor of Philosophy.

A preliminary report of this study was made at the meetings of the American Society of Biological Chemists at New Orleans in March, 1940 (A. Iliff, G. M. Kinsman, A. M. Duval and R. C. Lewis, J. Biol. Chem. 133: xlvii, 1940).

Received for publication, Aug. 31, 1942.

†This author's participation in this study and its publication have been approved by the Oklahoma Agricultural Experiment Station.

TABLE I

PERCENTAGE CHANGES AT DIFFERENT RANGES OF ALTITUDE FROM THE HEAT PRODUCTION ESTABLISHED AT ELEVATIONS BETWEEN SEA LEVEL AND 1,000 FEET

(A SUMMARY OF THE LITERATURE)

	1896-1913				1913-1941			
	No. of reports			7	No. of reports			9
	Total No. of subjects			15	Total No. of subjects			22
	RANGE IN ALTITUDE IN FEET ABOVE SEA LEVEL							
	1,000 TO 5,000	5,000 TO 7,500	7,500 TO 10,000	10,000 TO 15,000	1,000 TO 5,000	5,000 TO 7,500	7,500 TO 10,000	10,000 TO 15,000
Percentage changes in energy production	−11	− 7	− 8	− 7	−1	− 8	± 0	−15
	− 7	− 7	− 6	± 0	±0	− 2	+ 2	−11
	− 4	− 3	− 4	+ 3	+1	− 2	+ 4	− 4
	± 0	± 0	+ 5	+ 6		± 0	+ 5	+ 3
	+ 1	± 0	+ 9	+ 6		± 0	+10	+ 3
	+ 2	+ 1	+12	+ 9		+ 1	+16	+ 4
	+ 3	+ 1	+21	+10		+ 2		+ 5
	+ 4	+ 1		+12		+ 2		+ 5
	+ 4	+ 2		+15		+ 2		+ 5
	+ 9	+ 4		+16		+ 3		+ 6
		+ 9		+20		+ 3		+10
		+ 9		+43		+ 3		+10
				+45		+ 4		+11
				+45		+ 5		+12
				+86		+ 5		+13
						+ 5		+13
						+ 8		+14
						+ 8		+16
						+ 9		+17
						+14		+18
								+22
Range of percentage change	−11 to + 9	− 7 to + 9	− 8 to +21	− 7 to +86	−1 to +1	− 8 to +14	± 0 to +16	−15 to +22

Observations made during balloon ascents and in pneumatic chambers have also been used to study the effect of decreased barometric pressure on energy metabolism. The results of such experiments are not pertinent in relation to the problem under consideration, because the factor of acclimatization has entered into only a few of the investigations, and the purpose of the present study has been to determine whether altitude has a significant effect on the basal metabolism of acclimatized individuals.

Although this review of the literature reveals that change of altitude up to 10,000 feet has no significant effect on basal metabolism, the contrary conclusion has been drawn from a few studies[20, 21, 22] made at single elevations. In a later publication[23] the validity of such a conclusion will be questioned. However, inasmuch as these reports have appeared in the literature, the problem has been subjected to further investigation.

Experimental Procedure.—Although the primary purpose of this study was to determine whether the normal values of basal metabolism for children established at the altitude of Denver could be used as standards at lower altitudes, it was impracticable to use children as subjects of investigation. Accordingly, the study was made on seven adults. The basal metabolism of all seven subjects (two men and five women) was measured at Denver, Colo. (altitude, 5,280 feet) and at Stillwater, Okla. (altitude, 910 feet), and four of the sub-

jects (one man and three women) were also studied at Eldora, Colo. (altitude, 8,720 feet). During the period of residence at each place, no subject changed from that altitude by more than 1,000 feet for longer than one hour.

Three of the subjects (L., D., and I.) had been residents of Denver for at least ten years and had not been to an altitude lower than 5,000 feet for three months or longer before the first series of determinations was made at Denver. Between the completion of this initial period and the determinations in Stillwater, Subject L. spent four days at an altitude of about 900 feet but returned to Denver for 5 days just prior to leaving for Oklahoma. Subjects D. and I. remained in Denver until their departure for Stillwater. The other four subjects (N., K., H., and B.) had been residents in the Middle West for at least two years and had not been to an altitude higher than 1,000 feet during the three months before the first determinations were made on them in Stillwater.

After the initial period in Denver (Denver I), the three subjects, L., D., and I., motored to Stillwater on May 15 and 16 and remained there for twenty-four days. During their stay, basal metabolism determinations and blood studies were made on them and on the four residents of Stillwater. On June 10 the three subjects (L., D., and I.) returned to Denver where further determinations were made for a period of several weeks (Denver II). Subject H. came to Denver on July 1, and observations were made on her over a period of eight months while she remained at the higher altitude. On July 23 Subject K. arrived in Denver where determinations were made on her until August 5. These two latter subjects had remained in Oklahoma until their departure for Denver. In December, Subjects N. and B. came to Denver for determinations during a period of eleven days. They had been residing in Illinois at an altitude of about 700 feet since the end of the observations on them in Stillwater. Even though these studies on Subjects N., K., H., and B. constituted the first period of observations on them in Denver, this period has been designated, for the sake of clarity, as Denver II in the report to follow. Thus, for all seven subjects, the period known as Denver II indicates one following a change from a relatively low to a higher altitude.

Subjects D., I., and K. went to Eldora, Colo. on August 5 and were joined there by Subject L. on August 7. Determinations were made on all of them until August 21 when they returned to Denver where further observations were made in a final period at Denver (Denver III).

During the course of this study the living habits of the subjects were not materially changed at the different places. Indeed, the body weight and general physical condition of each subject varied little except for Subject L. who was ill just before the observations at Eldora. This illness occurred during a non-experimental period in Denver, and the weight of this subject remained relatively low throughout the period at Eldora and the final period at Denver.

The determinations of basal metabolism were made by the same individuals with the same closed circuit apparatus, a No. M 84 McKesson Metabolor, at all altitudes. From five to thirteen determinations were made on each subject in the initial periods, whether at Denver or at Stillwater. After each change in altitude, except during the third period in Denver, almost daily determinations were made for ten or more days. The subjects had fasted for at least twelve

hours and had rested on a comfortable bed in the laboratory for forty-five minutes before the first test to measure oxygen consumption was performed. In six instances only two tests were procured, since the variation in oxygen consumption from the mean was no greater than 2 per cent. In all other instances at least three tests of four minutes each or more were obtained. If the oxygen consumption of any one of the three tests showed a deviation of more than 4 per cent from the mean, another test was procured. When more than three tests were made, the deviation from the mean seldom exceeded 5 per cent. The mean value for oxygen consumption was used to calculate the heat production. As the respiratory quotient was not determined, 4.825 was used as the calorific value for each standard liter of oxygen. The basal metabolism calculated by the use of this factor is reported as calories per hour per square meter of body surface. The surface area values were obtained from a nomogram constructed by Wilson and Wilson[24] from the Du Bois and Du Bois height-weight formula.[25]

Eleven alcohol check determinations were made at intervals during the study, and these gave an average value of 0.4 per cent above the theoretical oxygen consumption with a range of from -1.5 to +2.9 per cent.

Blood studies made on each subject appear in a separate report by Lewis, Iliff, Duval, and Kinsman.[26] The values obtained for number of red cells, hemoglobin content, and packed red cell volume show that physiologic adjustment to altitude occurred comparatively early in the period of residence following each change in altitude.

EXPERIMENTAL RESULTS

The results of the individual determinations of basal metabolism are not presented here but may be found elsewhere.* For the most part, the values for each subject showed comparatively little variation in each period. However, it was found that on the first two days for Subjects L. and D. at Stillwater, on the first day for Subject I. at Stillwater, and on the first day for Subject H. at Denver the values for basal heat production (calories per hour per square meter) were markedly greater than those obtained subsequently during that period. Since these six high values were obtained in each case at the beginning of a period following a change in altitude and were distinctly out of line, they have been omitted in the calculation of the averages for the periods in which they occurred. However, even if these high results had been included, the means for these periods would not have been materially changed. Since in three instances the high initial values were observed on subjects who had moved from a higher to a lower altitude and in one case on a subject who had moved in the opposite direction, it is unlikely that the direction of change of altitude is the factor which caused these variations.

Table II shows the mean values for the calories per hour per square meter of body surface for the different periods as well as the standard errors of the means. From the latter values it may be observed that the means are reliable in all cases. Subjects N., I., K., H., and B show a close agreement in the means for the three altitudes. The values for Subject L. averaged 31.7 calories per

*The data of individual basal metabolism tests may be found in Part I, Table 2, of the ... [illegible] ...erta Iliff, "The Effect of Altitude on Certain Physiological Functions of ... which is on deposit in the Charles Denison, M.D., Memorial Library, Uni- ... School of Medicine, Denver. Microfilm copy of the table (pp. 19 to 32, incl.) may be obtained from the Librarian of the University of Colorado, Boulder, Colo.

TABLE II

NUMBER OF TESTS AND MEAN VALUES OF CALORIES PER HOUR PER SQUARE METER ± THE STANDARD ERROR OF THE MEAN FOR EACH SUBJECT AT EACH PERIOD

SUBJECT	DENVER I		STILLWATER		DENVER II		ELDORA		DENVER III	
	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF	NO. OF TESTS	MEAN ± THE STANDARD ERROR, THEREOF
N. (♂)			8	32.9 ± 0.36	10	33.5 ± 0.36				
L. (♂)	10	31.7 ± 0.27	15	34.4 ± 0.24	10	34.2 ± 0.24	10	34.2 ± 0.20	4	34.0 ± 0.55
D. (♀)	10	34.4 ± 0.45	12	32.0 ± 0.28	17	32.7 ± 0.27	11	33.1 ± 0.45	7	32.5 ± 0.53
L. (♀)	10	31.8 ± 0.39	11	31.8 ± 0.36	13	31.1 ± 0.21	13	31.5 ± 0.21	4	31.5 ± 0.56
K. (♀)			13	31.6 ± 0.42	7	31.4 ± 0.31	13	31.3 ± 0.34	3	30.5 ± 0.01
H. (♀)			5	30.5 ± 0.44	15	30.4 ± 0.33				
R. (♀)			7	31.9 ± 0.21	11	32.4 ± 0.24				

hour per square meter in the initial period in Denver and from 34.0 to 34.4 calories in the four subsequent periods. The values for Subject D. averaged 34.4 calories per hour per square meter in the first period in Denver, but the subsequent means were all lower and varied between 32.0 and 33.1 calories. It should be noted that the changes after the initial period for Subjects L. and D. are in the reverse direction from one another.

TABLE III

SIGNIFICANCE OF THE DIFFERENCE BETWEEN MEANS OF BASAL METABOLISM FOR EACH SUBJECT AT DIFFERENT PERIODS AS INDICATED BY THE RATIO OF THE DIFFERENCE BETWEEN THE TWO MEANS IN EACH CASE TO THE STANDARD ERROR OF THE DIFFERENCE

DATA USED	$\frac{m_1 - m_2}{\sigma_D}$* FOR SUBJECTS						
	N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Denver I and Stillwater		7.6†	4.5†	0.0			
Denver II and Stillwater	1.1	0.6	1.8	1.7	0.5	0.3	1.6
Denver III and Stillwater		0.7	0.8	0.5	2.6		
Denver I and Denver II		6.9†	3.2†	1.7			
Denver I and Denver III		3.7†	2.7	0.6			
Denver II and Denver III		0.4	0.4	0.6	2.7		
Denver I and Eldora		7.2†	2.0	0.8			
Denver II and Eldora		0.2	0.7	1.2	0.2		
Denver III and Eldora		0.3	0.8	0.0	2.2		
Stillwater and Eldora		0.9	2.0	0.8	0.7		

*The values used in the calculation of this formula are: m_1 = the mean obtained in one period, m_2 = the mean obtained in the other period, σ_D = the standard error of the difference or $\sqrt{\sigma^2_{m_1} + \sigma^2_{m_2}}$, where σ_{m_1} = the standard error of the mean of the values in one period and σ_{m_2} = the standard error of the mean of the values in the other period.

†The values so indicated are significant because the difference in the means is 3.0 or more times the standard error of the difference between the means.

Table III has been constructed to show whether there are any significant differences between the averages for the calories per hour per square meter of body surface obtained in the various experimental periods. The value given in each case is the ratio of the difference between two means to the respective standard error of this difference.[27] It may be seen that the differences between the basal heat production for any two periods are not significant for Subjects N., I., K., H., and B. On the other hand, the values for Subject L. show a significant difference between the initial period at Denver and each of the other periods. Similarly, the values for Subject D. indicate a significant difference between the basal heat production for the first period in Denver and those for the period in Stillwater and for the second period in Denver, respectively. However, when the comparison does not include the first period in Denver, it may be seen that the differences between the means of any other two periods for Subjects L. and D. are not significant. Since similar significant differences to those noted for Subjects L. and D. between the means of the initial period at Denver and those found at other altitudes are shown by these subjects between the means for the first period in Denver and for one or more subsequent periods at this same place, it is evident that factors other than altitude are responsible for the aberrant findings for these subjects in the initial period at Denver. Accordingly, it may be concluded that change in altitude up to 8,720 feet had no effect on the basal metabolism of any of the subjects studied.

In the present study lower values for intraindividual variability were found than those reported by Berkson and Boothby.[28] When the composite data for all periods on men and women, respectively, were treated by the procedure used by these authors on their similar data, the values for one standard deviation were found to be 1.27 calories per hour per square meter of body surface for the men and 1.19 calories for the women. Berkson and Boothby found 1.33 calories for men and 1.61 calories for women. Table IV, which gives the number of tests and the value for one standard deviation from the mean for each subject in each period as well as for the entire study, shows the greatest variability for any one period for the men to be 1.09 calories for Subject N. at Denver and for the women, 1.44 calories for Subject K. at Stillwater and for Subject D. at Eldora. Even when the calculation is made on all determinations of each subject, the highest values found are 1.30 calories on Subject L. for the men and 1.42 calories on Subject D. for the women. All of these values show a relatively low intraindividual variability.

TABLE IV

NUMBER OF TESTS AND VALUES FOR ONE STANDARD DEVIATION (σ)* FROM THE MEAN VALUE FOR CALORIES PER HOUR PER SQUARE METER OF BODY SURFACE OF EACH SUBJECT FOR EACH EXPERIMENTAL PERIOD

PERIOD	N. (♂)		L. (♂)		D. (♀)		I. (♀)		K. (♀)		H. (♀)		B. (♀)	
	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ	NO. OF TESTS	σ
Denver I			10	0.82	10	1.35	10	1.18						
Stillwater	8	0.98	15	0.89	12	0.90	11	1.14	13	1.44	5	0.88	7	0.49
Denver II	10	1.09	10	0.65	17	1.08	13	0.73	7	0.74	15	1.25	11	0.73
Eldora			10	0.61	11	1.44	13	0.66	13	1.19				
Denver III			4	0.95	7	1.30	4	0.86	3	0.09				
All Tests	18	1.08	49	1.30	57	1.42	51	0.97	36	1.21	20	1.17	18	0.69

*The formula used for this calculation was that of Boothby, Berkson and Dunn[29] $\sigma = \sqrt{mS - M^2}$, where mS is the mean of the squares of the differences of the observed values from the mean value of the individual under consideration and M is the mean of these differences.

Although the primary purpose of this investigation was to determine the effect of altitude on basal metabolism, the nature of the study makes possible some observations on geographic location. Talbot, Wilson, and Worcester[30] believe that this is one of the factors which affects the basal heat production, and Talbot and Talbot[31] suggest that the principal reason for the disagreement between the standards of basal metabolism for children established by them in Boston and those reported for the children of the Child Research Council at Denver[1] is the difference in geographic location between the two cities. Boston is 2.8° farther north than Denver, and differences in latitude of this same order are found between the places at which the present study was made. Eldora and Denver are 3.0° and 2.6°, respectively, farther north than Stillwater. However, no evidence has been obtained in the present study which would indicate that differences in latitude of this magnitude cause significant differences in basal heat production. Thus, no confirmation can be given here to support the Talbots' contention that the disagreement between the standards of basal metabolism for children established by them in Boston

and by the studies of the Child Research Council in Denver is due to the difference in geographic location.

Whether the illness of Subject L. in Denver just prior to the experimental period in Eldora had any effect on the results obtained on him at the higher altitude might be questioned. However, the fact that the basal heat production in his case remained unchanged, just as it did in the other Subjects (D., I., and K.) who were also studied at Eldora, indicates that the illness had no significant effect.

In conclusion, it may be well to comment upon whether the question which prompted the present study has been answered; namely, whether the standards of basal metabolism established on children of the Child Research Council at Denver may be used at other altitudes. As has been explained earlier in this paper, practical considerations necessitated the use of adults as subjects in this investigation. However, there appears to be no valid reason for expecting a difference between the effect of altitude on children and on adults. Accordingly, since change of altitude did not affect the basal metabolism of the adults studied, the standards[1, 32] for the basal metabolism of children established at Denver may be considered reliable for use at altitudes below 9,000 feet.

SUMMARY

1. The basal metabolism of seven adults (five women and two men) was determined at Denver, Colo. (altitude, 5,280 feet) and at Stillwater, Okla. (altitude, 910 feet). Four of the subjects (three women and one man) were also studied at Eldora, Colo. (altitude, 8,720 feet).

2. The subjects remained at each altitude sufficiently long to become acclimatized as judged by certain observations made on the blood for the measurement of physiologic adaptation. During the period of adjustment to the changes in altitude, except after the return to Denver from a higher altitude, determinations of basal metabolism were made almost daily and also for some days after acclimatization had been accomplished.

3. Change in altitude within the limits of the elevations studied did not affect the basal metabolism.

4. Changes in geographic location within the limits and under the conditions experienced in this study had no effect on the basal metabolism.

5. A critical review of the literature shows that change in altitude at levels below 9,000 feet does not affect the basal metabolism and this view is confirmed by the present study. Thus, it may be concluded that for elevations below this level the standards[1, 32] for the basal metabolism of children established by the investigations of the Child Research Council at Denver are reliable.

REFERENCES

1. Lewis, R. C., Kinsman, G. M., and Iliff, A.: The Basal Metabolism of Normal Boys and Girls From Two to Twelve Years Old, Inclusive, Am. J. Dis. Child. 53: 348, 1937.
2. Schumberg, H., and Zuntz, N.: Zur Kenntnis der Einwirkungen des Hochgebirges auf den menschlichen Organismus, Arch. f. d. ges. Physiol. 63: 461, 1896.
3. Loewy, A., Loewy, J., and Zuntz, L.: Ueber den Einfluss der verdünnten Luft und des Höhenklimas auf den Menschen, Arch. f. d. ges. Physiol. 66: 477, 1897.
4. Jacquet, A., and Stähelin, R.: Stoffwechselversuch im Hochgebirge, Arch. f. exper. Path. u. Pharmakol. 46: 274, 1901.

5. Durig, A.: Beiträge zur Physiologie des Menschen im Hochgebirge, Arch. f. d. ges. Physiol. 113: 213, 1906.
6. Loewy, A.: Die Gase des Korpers und der Gaswechsel, Oppenheimer's Handbuch der Biochemie des Menschen und der Tiere, Vienna, von Gustav Fischer 4: 220, 1911.
7. Zuntz, N., Loewy, A. Müller, F., and Caspari, W.: Höhenklima und Bergwanderungen in ihrer Wirkung auf den Menschen, Berlin, 1906, Bong and Co., Cited by Loewy.[6]
8. Hasselbalch, K. A., and Lindhard, J.: Analyse des Höhenklimas in seinen Wirkungen auf die Respiration, Skandinav. Arch. f. Physiol. 25: 361, 1911.
9. Durig, A., Zuntz, N., and von Schrötter, H.: Beobachtungen über die Wirkung des Höhenklimas auf Teneriffa, Biochem. Ztschr. 39: 435, 1912.
10. Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C.: Physiological Observations Made on Pike's Peak, Colorado, With Special Reference to Adaptation to Low Barometric Pressures, Phil. Tr. Roy. Soc. Lond. B 203: 185, 1913.
11. Liljestrand, G., and Magnus, R.: Die Wirkung des Kohlensäurebades beim Gesunden nebst Bemerkungen über den Einfluss des Hochgebirges, Arch. f. d. ges. Physiol. 193: 527, 1922.
12. Schneider, E. C.: The Respiratory Exchange and Alveolar Air Changes in Man at High Altitudes, Am. J. Physiol. 65: 107, 1923.
13. Barcroft, J., Binger, C. A., Bock, A. V., Doggart, J. H., Forbes, H. S., Harrop, G., Meakins, J. C., and Redfield, A. C.: Observations Upon the Effect of High Altitude on the Physiological Processes of the Human Body, Carried Out in the Peruvian Andes, Chiefly at Cerro dePasco, Phil. Tr. Roy. Soc. Lond. B 211: 351, 1921-1923.
14. Kestner, O., Dannmeyer, F., Peemöller, F., and Liebeschütz-Plaut, R.: Die Heilwirkung des Höhenklimas, Klin. Wchnschr. 4: 910, 1925.
15. Herxheimer, H., Wissing, E., and Wolff, E.: Spätwirkungen erschöpfender Muskelarbeit auf den Sauerstoffverbrauch. II. Versuche im Höhenklima, Ztschr. f. d. ges. exper. Med. 52: 447, 1926.
16. Kestner, O., Peemöller, F., and Schadow, H.: Ergebnisse einer Klimaexpedition nach Teneriffa, Arch. f. d. ges. Physiol. 217: 473, 1927.
17. Kestner, O., and Schadow, H.: Strahlung, Atmung und Gaswechsel. Versuche am Jungfraujoch, Arch. f. d. ges. Physiol. 217: 492, 1927.
18. Grollman, A.: Physiological Variations of the Cardiac Output of Man. VII. The Effect of High Altitude on the Cardiac Output and Its Related Functions: An Account of Experiments Conducted on the Summit of Pike's Peak, Colorado, Am. J. Physiol. 93: 19, 1930.
19. Asmussen, E., and Consolazio, F. C.: The Circulation in Rest and Work on Mount Evans (4,300 M.), Am. J. Physiol. 132: 555, 1941.
20. McCrery, J., and Wolf, M. M.: Basal Metabolism of Normal College Women at an Altitude of 3,200 Feet, J. Home Econ. 30: 583, 1938.
21. McCrery, J., Wolf, M. M., and Bavousett, N. D.: Studies in Basal Metabolism of Normal Women, J. Home Econ. 32: 562, 1940.
22. McKittrick, E. J.: Basal Metabolism of Wyoming University Women, J. Nutrition 11: 319, 1936.
23. Lewis, R. C., Iliff, A., and Duval, A. M.: Further Consideration of the Effect of Altitude on Basal Metabolism. A Study on Young Women Residents of Denver, J. Nutrition. In press.
24. Wilson, C. M., and Wilson, D.: The Determination of the Basal Metabolic Rate and Its Value in Diseases of the Thyroid Gland, Lancet 2: 1042, 1920.
25. Du Bois, D., and Du Bois, E. F.: Clinical Calorimetry. X. Formula to Estimate the Approximate Surface Area if Height and Weight Be Known, Arch. Int. Med. 17: 863, 1916.
26. Lewis, R. C., Iliff, A., Duval, A. M., and Kinsman, G. M.: The Effect of Change of Altitude on the Blood of Human Subjects, J. Lab. & Clin. Med. 28: 860, 1943.
27. Peters, C. C., and van Voorhis, W. R.: Statistical Procedures and Their Mathematical Bases, New York, 1940, McGraw-Hill Book Co., p. 162.
28. Berkson, J., and Boothby, W. M.: Studies of the Energy of Metabolism of Normal Individuals: The Interindividual and Intraindividual Variability of Basal Metabolism, Am. J. Physiol. 121: 669, 1938.
29. Boothby, W. M., Berkson, J., and Dunn, H. L.: Studies of the Energy of Metabolism of Normal Individuals: A Standard for Basal Metabolism With a Nomogram for Clinical Application, Am. J. Physiol. 116: 468, 1936.
30. Talbot, F. B., Wilson, E. B., and Worcester, J.: Basal Metabolism of Girls, Am. J. Dis. Child. 53: 273, 1937.
31. Talbot, F. B., and Talbot, N. B.: Disorders of Internal Gland Secretion in Children, J. Pediat. 10: 533, 1937.
32. Lewis, R. C., Duval, A. M., and Iliff, A.: Standards for the Basal Metabolism of Children From Two to Fifteen Years Old, Inclusive, J. Pediat. In press.

THE EFFECT OF CHANGE OF ALTITUDE ON THE BLOOD OF HUMAN SUBJECTS*

ROBERT C. LEWIS, PH.D., ALBERTA ILIFF, PH.D., AND ANNA MARIE DUVAL, PH.D., DENVER, COLO., AND GLADYS M. KINSMAN,† PH.D., STILLWATER, OKLA.

IN CONJUNCTION with an investigation to determine the influence of altitude on the basal metabolism of adult human subjects, studies on their blood were conducted primarily as a means of ascertaining when physiologic adjustment to the changes in altitude occurred. These studies consisted of the determination of the number of red cells, the hemoglobin content, the packed red cell volume, and the specific gravity. Although it has often been observed that physiologic alterations in the blood occur with changes in altitude, seldom has such an opportunity as the one encountered in this study been afforded to observe the effects of altitude on the same individuals by identical techniques performed by the same workers and with almost no change in the living habits of the subjects.

EXPERIMENTAL PROCEDURE

The subjects of the study were five women and two men, and the altitudes at which the observations were made were 910 feet, 5,280 feet, and 8,720 feet above sea level. Only four of the subjects were studied at the highest altitude. The conditions under which the experiments were conducted are described in detail in our report on the effect of altitude on basal metabolism.[1]

Freely flowing blood was collected from the fingertip directly into small vials in which three drops of 0.5 per cent solution of heparin had been allowed to dry. The blood was usually drawn before breakfast, but occasionally it was taken at other times of day than early in the morning. By making a relatively deep puncture, it was possible to collect readily from six to ten drops (approximately 0.5 c.c.) of blood, and this gave adequate amounts for the determination of the number of red cells, the hemoglobin content, the packed red cell volume, and the specific gravity.

The red cell counts were made on at least two dilutions from each sample of blood. Hayem's solution was used in all cases. The pipettes and the Levy-Hausser counting chambers had been certified by the Bureau of Standards. More than 1,000 cells were counted from each pipette, and the mean value of the number of red cells per cubic millimeter for the two dilutions has been used.

---

*From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver, and the Oklahoma Agricultural Experiment Station, Stillwater.

This report is taken from the dissertation submitted by Alberta Iliff to the Faculty of the Graduate School of the University of Colorado, June, 1942, in partial fulfillment of the requirements for the degree Doctor of Philosophy.

A preliminary report of this study was made at the meetings of the American Society of Biological Chemists at New Orleans in March, 1940 (Kinsman, G. M., A. M. Duval, A. Iliff and R. C. Lewis, J. Biol. Chem. 133: liii, 1940).

Received for publication, Aug. 31, 1942.

†This author's participation in this study and its publication have been approved by the Oklahoma Agricultural Experiment Station.

The hemoglobin content was determined by the acid hematin method. The Klett hemoglobinometer used for all readings was calibrated by comparison with the Van Slyke-Neill[2] manometer method of gas analysis for the determination of the oxygen-carrying capacity of the hemoglobin. In order to assure a constancy of light, the same daylight lamp was used at all altitudes, and a distance of 12 cm. from the light to the tubes was always maintained. Immediately after introducing the blood into the 0.1 N HCl, the solution was shaken for 3.75 minutes, and the readings were made within the following two minutes. This procedure had been found to give values very close to those obtained by the Van Slyke-Neill method when the conversion factor of 1.34 c.c. of oxygen for 1 Gm. of hemoglobin was employed. Two determinations that checked within 2 per cent were obtained on each sample of blood. The mean of these values has been used as the hemoglobin content of the blood.

The volume of packed red cells was determined by the use of Van Allen[3] hematocrit tubes. Instead of the clips or rubber bands customarily used to seal the lower end of the capillary tube, melted beeswax was employed for this purpose. The introduction of blood into the capillary tube was accomplished by drawing the blood up to the 100 per cent mark. After wiping the excess blood from the tip with a gauze sponge, the column of blood was carefully drawn up the capillary tube about ½ cm. Melted beeswax was then applied to the lower end of the tube with a camel hair brush and allowed to harden for three minutes. The tube was centrifuged at 2,500 revolutions per minute until there was no further change in the volume of the packed red cells. After centrifugation, the total volume of blood read at or very slightly below the 100 per cent mark, which showed that the air introduced into the lower end of the tube had escaped completely and that no blood had been lost. Triplicates from each sample of blood were run and usually the readings for packed red cell volume in all three tubes showed agreement. Unless two of the readings were within 2 per cent of one another, the determinations were repeated. The mean value for the two or three acceptable determinations has been used.

The specific gravity was determined by the use of the falling drop apparatus of Barbour and Hamilton,[4, 5] which was standardized by the use of solutions of potassium sulfate with specific gravities of 1.0551, 1.0645 and 1.0743. In the determination of the specific gravity, triplicate readings of the rate of fall of blood and of standard, respectively, were considered acceptable when they agreed within 3 per cent. The means of these values for the blood and for the standard were used in the calculation of the specific gravity of the blood.

In addition to the blood studies, measurements of pulse rate and blood pressure were made several times on each subject at each altitude. Since the results were normal in all cases and no significant variation occurred on changing altitude, the data will not be reported.

RESULTS AND DISCUSSION

Table I gives the results of determinations of number of red cells, hemoglobin content, packed red cell volume, and specific gravity on bloods collected in Denver at different times of day on Subjects D., I., and L. at a time when they were thoroughly acclimatized. It shows that in none of these determinations

TABLE I

COMPARISON OF THE DETERMINATIONS OF THE NUMBER OF RED CELLS, THE AMOUNT OF HEMOGLOBIN, THE PACKED RED CELL VOLUME, AND THE SPECIFIC GRAVITY OF THE BLOOD OBTAINED ON THE SAME DAY UNDER FASTING AND NONFASTING CONDITIONS

SUBJECT	RED CELLS (MILLIONS PER CU. MM.)		HEMOGLOBIN (GM. PER 100 ML.)		CELL VOLUME (VOLUMES PER CENT)		SPECIFIC GRAVITY	
	FASTING	NON-FASTING	FASTING	NON-FASTING	FASTING	NON-FASTING	FASTING	NON-FASTING
D. (♀)	5.30	5.26	14.6	14.4	45.4	43.0	1.0620	1.0571
	5.24	5.16	14.5	14.4	46.0	46.1	1.0605	1.0602
	4.99	4.96	14.4	13.9	44.6	43.9	1.0627	1.0616
I. (♀)	5.32	5.26	14.9	15.0	46.0	45.9	1.0608	1.0663
	5.32	5.33	15.8	15.0	46.1	45.4	1.0627	1.0618
	5.03	5.01	15.0	14.9	45.0	43.1	1.0594	1.0631
	5.35	5.27	15.6	15.0	47.3	45.3	1.0620	1.0623
L. (♂)	6.08	6.28	17.0	17.4	50.6	51.3	1.0671	1.0664
	6.30	6.33	17.1	16.6	48.8	48.8	1.0675	1.0673
	6.26	6.14	17.0	16.2	50.4	50.9	1.0673	1.0669

is there a significant difference between the values obtained on fasting morning bloods and on those collected later during the day. Consequently, the few determinations that were made on blood collected under other than fasting conditions have been included in the data used.

Tables II, III, and IV give the means for the number of red cells, the hemoglobin content and the packed red cell volume, respectively, for the samples of blood taken at each altitude from each subject. In calculating these means, all values have been used except those obtained on the first three days after a change in altitude. The three-day interval was chosen arbitrarily, since the

TABLE II

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE NUMBER OF RED CELLS OF THE BLOOD. (FIRST THREE OF THESE VALUES EXPRESSED AS MILLIONS OF RED CELLS PER CUBIC MILLIMETER OF BLOOD)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	7	9	10	6	4	3
	Mean	5.38	5.56	4.62	4.82	4.47	4.72	4.55
	Standard error	0.064	0.026	0.034	0.081	0.069	0.100	0.029
	Standard deviation	0.112	0.065	0.098	0.244	0.153	0.172	0.041
	Coefficient of variation (per cent)	2.1	1.2	2.1	5.1	3.4	3.6	0.9
Denver (Altitude, 5,280 feet)	No. of tests	6	8	20	19	7	12	6
	Mean	5.84	6.30	4.79	5.08	4.83	4.64	4.87
	Standard error	0.113	0.092	0.044	0.032	0.062	0.083	0.037
	Standard deviation	0.253	0.244	0.194	0.137	0.153	0.274	0.083
	Coefficient of variation (per cent)	4.3	3.9	4.0	2.7	3.2	5.9	1.7
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		5.99	4.93	5.35	4.82		
	Standard error		0.063	0.028	0.040	0.032		
	Standard deviation		0.154	0.080	0.114	0.091		
	Coefficient of variation (per cent)		2.6	1.6	2.1	1.9		

TABLE III

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE HEMOGLOBIN CONTENT OF THE BLOOD (FIRST THREE OF THESE VALUES EXPRESSED AS GRAMS OF HEMOGLOBIN PER 100 MILLILITERS OF BLOOD)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	7	10	10	7	4	3
	Mean	14.05	15.70	13.44	13.85	12.86	12.75	12.33
	Standard error	0.098	0.210	0.086	0.189	0.270	0.459	0.249
	Standard deviation	0.170	0.516	0.258	0.568	0.662	0.795	0.352
	Coefficient of variation (per cent)	1.2	3.3	1.9	4.1	5.1	6.2	2.8
Denver (Altitude, 5,280 feet)	No. of tests	6	8	20	20	7	12	6
	Mean	16.28	16.78	14.10	15.18	14.13	12.92	13.00
	Standard error	0.086	0.147	0.110	0.077	0.174	0.158	0.188
	Standard deviation	0.192	0.390	0.478	0.336	0.425	0.524	0.420
	Coefficient of variation (per cent)	1.2	2.3	3.4	2.2	3.0	4.0	3.2
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		16.73	14.83	16.06	14.91		
	Standard error		0.171	0.212	0.274	0.289		
	Standard deviation		0.419	0.599	0.577	0.818		
	Coefficient of variation (per cent)		2.5	4.0	3.6	5.5		

TABLE IV

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE PACKED RED CELL VOLUME OF THE BLOOD (FIRST THREE OF THESE VALUES EXPRESSED AS VOLUMES PER CENT OF BLOOD)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	6	10	10	7	4	3
	Mean	45.52	47.70	41.98	41.29	39.59	39.20	38.43
	Standard error	0.655	0.276	0.355	0.447	1.058	0.641	1.761
	Standard deviation	1.134	0.462	1.065	1.341	2.590	1.111	2.490
	Coefficient of variation (per cent)	2.5	1.0	2.5	3.2	6.5	2.8	6.5
Denver (Altitude, 5,280 feet)	No. of tests	6	8	20	18	7	12	6
	Mean	47.02	50.64	43.32	45.58	41.20	39.58	39.33
	Standard error	0.316	0.459	0.273	0.366	0.470	0.443	0.631
	Standard deviation	0.707	1.215	1.192	1.508	1.152	1.470	1.410
	Coefficient of variation (per cent)	1.5	2.4	2.8	3.3	2.8	3.7	3.6
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		49.09	45.62	48.53	41.24		
	Standard error		0.661	0.812	0.516	0.710		
	Standard deviation		1.619	2.296	1.458	2.009		
	Coefficient of variation (per cent)		3.3	5.0	3.0	4.9		

changes observed in the different subjects showed no constancy with respect to the time of the beginning of the change or to its degree. In addition to the mean in each case, the tables give the standard error of the mean, the standard deviation from the mean, and the coefficient of variation. These statistical values show that there is relatively little scatter in the data obtained at any altitude for any of the subjects. When the means for each subject at the different altitudes are compared, it is seen that with an increase in altitude there were increases in eight instances for the number of red cells, in ten instances for the hemoglobin content, and in nine instances for the packed red cell volume out of eleven possibilities in each case. Thus, in the majority of instances adjustment to a higher altitude was manifested by increases in all three of these values.

Exceptions to the general rule that increases in the number of red cells, in the hemoglobin content, and in the packed red cell volume occur with an increase in altitude are to be found in some instances in the cases of Subjects H., K., and L. Practically no difference in the means occurred in any of these values when Subject H. moved from Stillwater (altitude, 910 feet) to Denver (altitude, 5,280 feet) or in the number of red cells or in the packed red cell volume when Subject K. went from Denver to Eldora (altitude, 8,720 feet). No reasons are evident for the inconsistencies found in these cases. When the mean values for Subject L. during the period at Eldora are compared with those obtained during the experimental periods in Denver, definite decreases in number of red cells and in packed red cell volume and practically no change in hemoglobin content are observed. An illness experienced by this subject during a nonexperimental period in Denver just prior to his departure for Eldora may

TABLE V

THE MEAN, THE STANDARD ERROR AND THE STANDARD DEVIATION OF THE MEAN, AND THE COEFFICIENT OF VARIATION FOR THE SPECIFIC GRAVITY OF THE BLOOD (FIRST THREE OF THESE VALUES EXPRESSED IN TERMS OF SPECIFIC GRAVITY)

		SUBJECTS						
		N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Stillwater (Altitude, 910 feet)	No. of tests	4	7	10	10	7	4	3
	Mean	1.0646	1.0660	1.0614	1.0610	1.0581	1.0594	1.0572
	Standard error	0.0024	0.0039	0.0009	0.0012	0.0040	0.0016	0.0007
	Standard deviation	0.0042	0.0096	0.0028	0.0035	0.0099	0.0028	0.0010
	Coefficient of variation (per cent)	0.4	0.9	0.3	0.3	0.9	0.3	0.1
Denver (Altitude, 5,280 feet)	No. of tests	6	8	19	16	7	12	6
	Mean	1.0656	1.0672	1.0611	1.0624	1.0607	1.0580	1.0580
	Standard error	0.0015	0.0034	0.0009	0.0008	0.0013	0.0005	0.0008
	Standard deviation	0.0033	0.0090	0.0039	0.0033	0.0032	0.0017	0.0017
	Coefficient of variation (per cent)	0.3	0.8	0.4	0.3	0.3	0.2	0.2
Eldora (Altitude, 8,720 feet)	No. of tests		7	9	9	9		
	Mean		1.0667	1.0629	1.0650	1.0633		
	Standard error		0.0032	0.0008	0.0013	0.0014		
	Standard deviation		0.0079	0.0022	0.0036	0.0040		
	Coefficient of variation (per cent)		0.7	0.2	0.3	0.4		

account for his failure to have attained higher means for the number of red cells, hemoglobin content, and packed red cell volume at the higher altitude. Four days before he went to the higher altitude, determinations on his blood showed that the number of red cells had fallen to 5.14 millions per cubic millimeter and that the hemoglobin content had dropped to 15.8 Gm. per 100 ml. of blood. Although recovery was rapid, as shown by the fact that six days later or two days after reaching the higher altitude the number of red cells had increased to 5.75 millions per cubic millimeter and the hemoglobin to 17.2 Gm. per 100 ml. of blood. the mean for these values and for packed red cell volume during the period at Eldora probably did not reach the height they would have attained, had this period not followed so closely upon his illness.

Table V gives the mean, the standard error and the standard deviation of the mean, and the coefficient of variation for the specific gravity of the blood at the different altitudes. For any given altitude very little intervariation or intravariation occurs. However, when one compares the mean of each subject at one altitude with that of the next higher altitude, it may be seen that in eight out of eleven possibilities slight but definite increases in specific gravity take place. Since the specific gravity of red blood cells is approximately 1.090 and that of serum 1.027,[6] each 5 per cent change in packed red cell volume would be expected to result in a parallel change of 0.003 in the specific gravity of the

TABLE VI

SIGNIFICANCE OF THE DIFFERENCE BETWEEN THE MEANS OBTAINED AT A HIGHER AND A LOWER ALTITUDE, RESPECTIVELY, FOR NUMBER OF RED CELLS, HEMOGLOBIN CONTENT, PACKED CELL VOLUME, AND SPECIFIC GRAVITY AS INDICATED BY THE RATIO OF THE DIFFERENCE BETWEEN THE TWO MEANS IN EACH CASE TO THE STANDARD ERROR OF THE DIFFERENCE

DIFFERENCES IN MEANS		$\frac{M_1 - M_2}{\sigma_D}$* FOR SUBJECTS						
FOR	OBTAINED AT	N. (♂)	L. (♂)	D. (♀)	I. (♀)	K. (♀)	H. (♀)	B. (♀)
Number of red cells	Denver and Stillwater	3.6†	7.7†	3.0†	3.0†	4.0†	−0.6	6.9†
	Eldora and Denver		−2.7	2.6	5.1†	−0.2		
	Eldora and Stillwater		6.4†	6.8†	5.8†	4.6†		
Hemoglobin content	Denver and Stillwater	17.2†	4.2†	4.7†	6.5†	4.0†	0.3	2.1
	Eldora and Denver		−0.2	3.1†	3.1†	2.3		
	Eldora and Stillwater		3.8†	6.1†	6.6†	5.2†		
Cell volume	Denver and Stillwater	2.0	5.5†	3.0†	7.4†	1.4	0.5	0.5
	Eldora and Denver		−1.9	2.7	4.7†	0.1		
	Eldora and Stillwater		1.9	4.1†	10.6†	1.3		
Specific gravity	Denver and Stillwater	0.4	0.2	−0.2	1.4	0.6	−0.9	0.7
	Eldora and Denver		−0.1	1.0	1.5	1.3		
	Eldora and Stillwater		0.1	1.1	2.4	1.1		

*The values used in the calculation of this formula are: m_1 = the mean obtained at the higher altitude, m_2 = the mean obtained at the lower altitude, σ_D = the standard error of the difference or $\sqrt{\sigma^2_{m_1} + \sigma^2_{m_2}}$, where σ_{m_1} = the standard error of the mean of the values at the higher altitude and σ_{m_2} = the standard error of the mean of the values at the lower altitude.

†The values so indicated are significant because the difference in the means is 3.0 or more times the standard error of the difference between the means.

whole blood. It is interesting to note that, except for Subjects D. and H. in changing from Stillwater to Denver and for Subject K. in going from Denver to Eldora, the alterations in specific gravity which do occur are in the direction and of the approximate magnitude to be expected from the changes in packed red cell volume shown in Table IV.

Table VI has been constructed to show whether the changes in the number of red cells, in the amount of hemoglobin, in the packed red cell volume, and in the specific gravity of the blood, which have been observed to occur with an increase in altitude, are significant. In eleven out of fifteen possibilities significant increases in the number of red cells and in the amount of hemoglobin occurred with an increase in altitude. Fewer marked changes were observed in packed red cell volume, for only three of the seven subjects showed significant increases with a change in residence from Stillwater to Denver, and only one of these showed a significant increase in changing from Denver to Eldora. No significant changes in specific gravity were found.

SUMMARY

1. Determinations of the number of red cells, the hemoglobin content, the packed red cell volume, and the specific gravity were made on the blood of seven adult subjects (five women and two men) at Stillwater, Okla. (altitude, 910 feet) and at Denver, Colo. (altitude, 5,280 feet). These same determinations were also made on the blood of one of the men and three of the women at Eldora, Colo. (altitude, 8,720 feet).

2. Although there was considerable variation in the amount of response of different individuals, and in a number of cases no significant change occurred, increases in the number of red cells, in the amount of hemoglobin, and in the packed red cell volume were found in the majority of instances with increases in altitude.

3. Definite but insignificant increases in specific gravity were found to occur in the majority of the cases with each increase in altitude. With few exceptions, these changes in specific gravity were in the direction and of the approximate magnitude to be expected from the alterations in packed red cell volume.

REFERENCES

1. Lewis, R. C., Iliff, A., Duval, A. M., and Kinsman, G. M.: The Effect of Change of Altitude on the Basal Metabolism of Human Subjects, J. Lab. & Clin. Med. 28: 851, 1943.
2. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, J. Biol. Chem. 61: 523, 1924.
3. Van Allen, C. M.: An Hematocrit Method, J. Lab. & Clin. Med. 10: 1027, 1925.
4. Barbour, H. G., and Hamilton, W. F.: The Falling Drop Method for Determining Specific Gravity, J. A. M. A. 88: 91, 1927.
5. Guthrie, C. C.: An Apparatus for Quickly Measuring the Specific Gravity of Body Fluids, J. Lab. & Clin. Med. 17: 1158, 1932.
6. Bodansky, M.: An Introduction to Physiological Chemistry, ed. 4, New York, 1938, John Wiley & Sons, Inc., p. 222.

LABORATORY METHODS

GENERAL

AN ELECTRONIC DEVICE FOR THE LOCATION OF METALLIC FRAGMENTS IN VIVO*

CON FENNING, M.D., SALT LAKE CITY, UTAH

MOORHEAD has called attention to a foreign body finder.[1] I wish to call attention to a simple electronic device consisting of a minimum of parts which can be used in a similar manner. It is self-contained in a portable case, measuring 6 by 6 by 6 inches and weighing 8 pounds. The circuit to be described may be so adjusted that it has discriminatory powers which may be of use in applications other than the localization of in vivo foreign bodies.

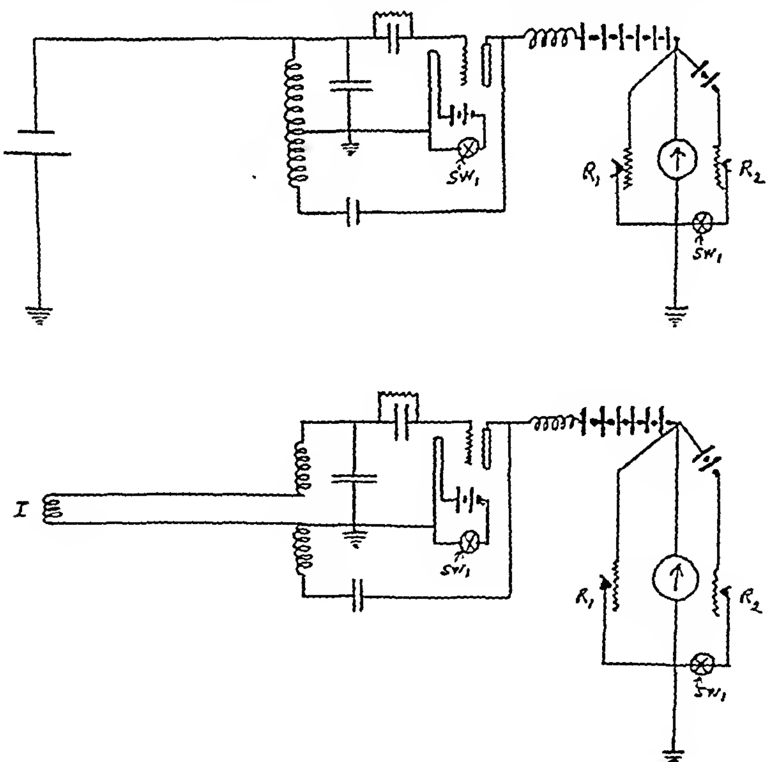


Fig. 1.

The device in question is based upon a modification of an electronic circuit previously described.[2] The schematic circuits here shown (Fig. 1) illustrate the

*From the Department of Pharmacology and Physiology, University of Utah School of Medicine, Salt Lake City.

Received for publication, June 12, 1942.

modification employed in order to convert the circuit to its present adaptation. From examination of the two circuits it is obvious that the insertion of inductance I with the elimination of the capacity pick-up plate represents the principal change.

Inductance I is incorporated in the circuit by way of a shielded conductor cable one yard in length. It is a small solenoid affixed to the tip of a locator probe 12 inches in length, having a diameter of 0.8 cm., and in the present model is constructed of fiber rod provided with a sterilizable glass covering. The circuit constants are so adjusted that when the locator tip is brought into the presence or in contact with metallic objects characteristic directional deflections occur on the part of the indicating member of the milliammeter placed in the plate circuit.

Table I shows the discriminatory direction deflections obtained when contact is made with the materials listed. Of all the metals listed, iron or alloys of iron gave positive deflections, whereas the remainder of the metals gave negative deflections. There is evidence that for equivalent surface areas soft iron has a greater deflection factor than steel. Similar trends are apparent for metals which cause negative deflections. On the other hand, if discrimination is not desired, the circuit constants may be so adjusted to provide positive or negative deflection for all metals. Experimental evidence indicates that it is possible to "peak" the circuit in such a manner that maximum deflection may be obtained for any one of the series of metals listed.

TABLE I

DIRECTION OF DEFLECTION (POSITIVE OR NEGATIVE)

Metals:	
Aluminum	-
Antimony	-
Brass	-
Chromium	-
Cobalt	-
Copper	-
Gold	-
Iron, soft	+
Lead	-
Magnesium	-
Mercury	-
Nickel	-
Silver	-
Sodium	-
Steel	+
Tin	-
Zinc	-
Nonmetals:	
Cement	+
Fiber	+
Glass	+
Mica	+
Paper	+
Wood	+
Solutions:	
Alcohol, absolute	+
Benzol	+
Chloroform	+
Ether	+
Kerosene	+
Water, distilled	+

A check of the maximum air distance between the tip of the locator and the objective for the recognition by demonstrable initial deflection was found to average 1 cm.; however, there existed some differences in this respect with each metal tested. The maximum air distance should be considered in a relative sense in view of the enhanced distance when using a larger probing inductance, and in view of the possibility of substituting a more sensitive meter in the plate circuit. On the other hand, a smaller probe possesses attributes not possessed by larger probes by virtue of its smaller size.

The surfaces of nonmetallic objects were tested, and the maximum air distance for a recognizable change was found to be very small—1 mm. or less—and in most instances contact was essential. In all objects or liquids tested a slight positive deflection occurred. As with the metals the deflection factor varied somewhat with each solution. When metallic objects were tested under water or other liquids, the directional changes noted on deflection were the same as those observed in air. The maximum water distance for recognizable initial change was at least as great as or greater than that found in air.

Experimental implantation of metallic iron and copper test objects (measuring 2 cm. by 2 cm. by 0.5 mm., 1 cm. by 1 cm. by 0.5 mm., 0.5 cm. by 0.5 cm. by 0.5 mm., and 0.25 cm. by 0.25 cm. by 0.5 mm.) was made in the dog. All implants of the above sizes were readily located beneath the skin—skin hair on or off, wet or dry. Deeper burial in the tissues beneath the surface of the skin were also readily located, provided the locator tip was within a distance of 1 cm. or less of the object. Similar implants placed in the abdominal cavity were also readily located.

Actual operation as a foreign body locator is as follows: Sw_1 is turned on. R_1 is rotated to introduce additional shunt resistance in parallel with the meter. The deflection of the meter is controlled to desired scale point by rotation of R_2. Locator is applied to region involved. On contact a small positive deflection occurs. The locator is then moved from one point to another, and any change in deflection, positive or negative, is noted. When such is manifested, the locator tip is manipulated in direction and depth in order to secure the maximum sustained deflection. When such is obtained, the tip of the locator is very near or in contact with the foreign metallic body. Maximum deflection results when the tip surface is parallel to the surface of the object and the minimum deflection results when the tip surface is at right angles to the surface of the object.

REFERENCES

1. Science, Suppl. 95: 8, 1942.
2. Fenning, C.: J. Lab. & Clin. Med. 22: 1279, 1937.

USE OF THE PHOTRONREFLECTOMETER FOR DETERMINATION OF THE OPTIMAL RATIO BETWEEN PNEUMOCOCCUS TYPE I SSS AND ANTIPNEUMOCOCCUS TYPE I RABBIT SERUM

Donald S. Martin, M.D., Durham, N. C.

INTRODUCTION

THE optimal proportions method of precipitin titration, introduced by Dean and Webb[1] in 1926, was based upon their observation that in a series of mixtures containing constant amounts of antiserum and varying amounts of antigen, the fastest precipitation occurred in that mixture which contained antigen and antibody in optimal proportions. Since this technique requires visual comparison of the relative reaction speeds in a number of tubes, its usefulness as a quantitative method of precipitin titration is limited. Consequently, more accurate evaluation of the quantitative relationships between antigen and antibody in the precipitin reaction[2] has resulted from the use of the objective quantitative chemical method of precipitate analysis described by Heidelberger and Kendall.[3]

Although the optimal proportions principle has been challenged on a theoretical basis by Heidelberger and Kabat,[4] the Dean-Webb procedure offers the only available means of determining separate antibody titers in sera containing more than one antibody when tested with solutions containing more than one antigen. Titration of individual antibodies in such a system is of great importance in the study of cross reactions, particularly in determining the proper absorbing dose of antigen. In absorbing an antiserum containing one or more antibodies with a solution containing multiple antigens, exhaustion of the homologous precipitins can be attained only by leaving a supernatant containing different concentrations of each antigen to interfere with subsequent tests for residue antibodies.

It was felt that the problem of precipitin titration in complex systems was of sufficient importance to justify the reinvestigation of the validity of the Dean-Webb optimal ratio. This paper describes a photoelectric method for recording the rate of precipitation and shows that the optimal proportions principle can be used for accurate precipitin titration. The application of the method to the titration of antibodies in complex antigen-antibody systems will be presented in a later paper. Since the photoelectric method has never before been applied to the problem of measuring precipitation rates in antigen-antibody mixtures, the technique is described in detail.

LITERATURE

Ramon[5] was the first to show that the antitoxin titer of a serum could be estimated by observing the first tube to flocculate in a series of tubes containing

*From the Departments of Bacteriology and Medicine, Duke University School of Medicine and Duke Hospital, Durham.

Aided by a grant from the John and Mary R. Markle Foundation.
Received for publication, June 25, 1942.

constant volumes of toxin and varying amounts of antitoxin. Dean and Webb[1] reversed the procedure, using a constant volume of serum and varying quantities of antigen. Marrack[6] has referred to these two methods as the alpha (constant antibody) and the beta (constant antigen) procedures. Brown[7] compared the two methods and introduced a third gamma procedure which was carried out after flocculation was complete in the beta series. The tubes were inverted twice, resulting in dispersion of the floccules in some of the mixtures. Brown concluded that there were three types of antigen-antibody reactions, which she described as follows: A type of reaction illustrated by the diphtheria toxin-antitoxin reaction, in which the alpha and beta flocculation zones coincided; that illustrated by the polysaccharide-antibody reaction, in which the alpha and beta methods yielded different flocculation zones but could be made to coincide by using the gamma technique; and that illustrated by the reaction between certain fractions of human serum and homologous antisera, in which the alpha and beta titrations differed and could not be made to agree by the gamma method.

The argument concerning the relationship of the alpha and beta optimal zones may have resulted from differences in opinion concerning the interpretation of the word "floccule." There is no logical basis for reasoning that the fastest reacting mixture in a constant antibody series should necessarily be the fastest reacting mixture in a constant antigen series. Flocculation, however, is a different phenomenon and is evidently related to the proportions of antigen and antibody in the mixture. This is suggested by Brown's[7] work showing that the floccules in certain mixtures could be dispersed after equilibrium had been established, bringing into agreement the different values obtained by the Ramon and Dean-Webb methods in the polysaccharide precipitin system.

The difficulties, inherent in the interpretation of results by either method, are illustrated by the work of Taylor,[8] who reported in 1931 that an optimal Dean-Webb ratio could be obtained for the horse serum antigen-antibody reaction, but that there was no optimal zone in the same system when titered by the Ramon technique. Increasing concentrations of antiserum caused only a speeding up of the reaction without any evidence of inhibition by excess antibody. In 1933, using horse serum and crystalline egg albumin as antigens, he[9] reversed his previous position and stated that the reaction was inhibited by antibody excess.

Although Dean and Webb stated that the optimal mixture was the fastest reacting mixture in a constant antibody-varying antigen series, the reactions in the tubes were described as proceeding from opalescence to turbidity, particulation, and finally flocculation. Since opalescence often appeared in several tubes simultaneously, they suggested that the optimal tube was detected easiest if the reaction were read shortly after particulation occurred, preferably at the time the particles were increasing in size. However, after equilibrium has been established in a constant antibody series, the greatest weight[1] and largest volume[10] of precipitate are found in mixtures containing excess antigen and not in the optimal reacting mixture. This emphasizes the fact that as the reaction progresses, there is a shift in particle size toward the region of antigen excess, suggesting that the mixtures should be compared as early as possible in the reaction.

Theoretical objections to acceptance of the Dean-Webb optimal proportions principle have been expressed by Heidelberger and Kabat[4] as follows: ". . . in a precipitation or agglutination reaction the factors influencing the velocity are imperfectly understood; (2) the position of the flocculation optimum is not independent of the dilution and varies whether the antigen or antibody be diluted; (3) in neither case is the optimum at the point of antibody exhaustion, so that the method gives no information as to the actual agglutinin or precipitin content of a serum."

Although the photoelectric method of measuring precipitation rates does not yield information concerning the actual speed of antigen-antibody union, differences in rate of formation of the light-reflecting precipitated particles can be determined and compared. Objections (2) and (3) are valid, since they refer to end points based on flocculation time and are subject to the differences in interpretation discussed above.

Certain technical difficulties are involved in preparing a series of mixtures for titration by either the Dean-Webb or Ramon method. For accurate visual comparison of the optimal and adjacent tubes, the mixtures should be made as nearly simultaneously as possible. As the ideal of simultaneous mixing is approached, accuracy in pipetting and mixing of the reagents must decrease. Conversely, precise measurement and thorough mixing cause a widening of the time interval between preparation of the various tubes in either series, creating difficulty in making satisfactory comparisons. Smith[11] discussed the problem in detail and suggested that the occurrence of double zones might be caused by improper mixing.

The method presented here avoids these difficulties, since each mixture is made separately and the speed of each reaction, measured by photoelectric means, is recorded permanently and is available for comparison at any time.

MATERIALS AND METHODS

Materials.—Pneumococcus type I polysaccharide was used in all experiments. The preliminary observations were made with a sample of dried polysaccharide furnished by Dr. A. F. Goebel and Dr. T. M. Rivers of the Rockefeller Institute for Medical Research. Another batch of material was obtained from the Lederle Laboratories through the courtesy of Dr. A. L. Joyner and Miss Frances L. Clapp. The polysaccharide was dissolved in sufficient 0.85 per cent saline (containing merthiolate 1:10,000) to make a concentration of 1 mg. per milliliter of solution.

The antiserum in all experiments was refined antipneumococcus type I rabbit serum furnished by the Lederle Laboratories. The antibody content of this serum, as determined in these laboratories, was 5.7 mg. antibody N per milliliter.

Apparatus.—Libby's[12] photronreflectometer was chosen because its usefulness in the precipitin reaction had been demonstrated by Libby[13, 14] and Bukantz, Cooper, and Bullowa[15] who showed that the pneumococcus polysaccharide content of a solution could be determined by observing the galvanometer deflection at a constant time interval after mixing. This instrument is constructed with a small black circular disk in the center of the photoelectric cell directly opposite the circular window of the chamber containing the mixture. Consequently, the

galvanometer reading is dependent upon the number of light rays deflected by particles in the chamber impinging on the periphery of the photoelectric cell.

Preliminary observations showed a short but definite lag in galvanometer response when a stable vaccine suspension was introduced into the apparatus. Before each experiment the galvanometer was adjusted to give a reading of 10 on the scale. Introduction of the clean empty chamber always caused a shift to a value between 2 and 3. The deflection of the empty chamber was determined before each experiment, and this value was subtracted from all readings made during the experiment.

Accuracy.—Sixteen determinations were made with a sample of diluted typhoid vaccine. Four different chambers were used, washed between each determination, and the readings were corrected for the deflection of the empty chamber. The highest deflection was 39.9, the lowest 38.3, the mean 39.0, and the standard deviation 0.40. Since the precipitation reaction involves two reagents and introduces errors in measurement, mixing, and timing, 10 separate experiments were done, using the same antigen and antibody solutions. Readings made five minutes after mixing showed a maximum of 56.6, a minimum 52.8, mean 54.0, and standard deviation 1.34.

Temperature Controls.—All experiments were done at room temperature (21° to 22° C.), the serum and antigen dilutions being kept in a room temperature water bath until used. However, it was observed that the temperature of the fluid in the chamber rose as much as 6° C. in five minutes if the apparatus had been used for seven or eight consecutive experiments. This was controlled partially by continuous passage of a stream of air through a tube inserted in one of the ventilating holes of the apparatus and disconnecting the machine from the electric current for a five-minute interval between experiments. The temperature rise was not eliminated but could be reduced to an almost constant value of 2.1° C. for each five-minute experiment.

Method of Mixing.—Mixing of antigen and antibody was accomplished by expelling 1.4 ml. of the antigen solution into a Wassermann tube containing 1.4 ml. of diluted serum. The mixture was drawn back into the pipette and expelled twice. The third filling of the pipette was transferred to the chamber previously placed in the apparatus.

Reading Times.—An assistant started a stop watch at the time the antigen solution was expelled into the serum. Galvanometer readings were made at ten-second intervals for the first minute, at fifteen-second intervals during the second minute, and at thirty-second intervals for the remaining three minutes. Except for the preliminary observations, all experiments were terminated five minutes after mixing.

Supernatant Examination.—After each experiment the chamber contents were poured into a Wassermann tube and placed in the icebox. After centrifugation the next morning, each supernatant was tested with three dilutions of antibody and three dilutions of antigen.

Calculations.—Each deflection was corrected by subtracting from it the deflection of the empty chamber. A second correction was made for the deflection caused by the control serum. Because of slight bubble formation, the introduction of control serum-saline mixtures caused an increase in deflection followed

by a slow drift back to a stabilized value in thirty to forty seconds. The control values at the appropriate time intervals were subtracted from all readings made during each antigen-antibody experiment.

EXPERIMENTAL

Effect of Flocculation on Galvanometer Deflection.—Since flocculation is followed by a settling out of the precipitate in the chamber, this experiment was planned to determine the effect of prolonged incubation on the galvanometer deflection. The data, obtained before the temperature control experiments were done, were not as accurate as those obtained later, but the results illustrated the limitations of the method after flocculation had occurred.

The mixtures were made in the usual way, but after recording the deflections for the usual five-minute periods, the contents of each chamber were poured into Wassermann tubes. At various intervals over a two-and-a-half-hour period, each mixture was shaken, placed in the chamber, and the deflection recorded.

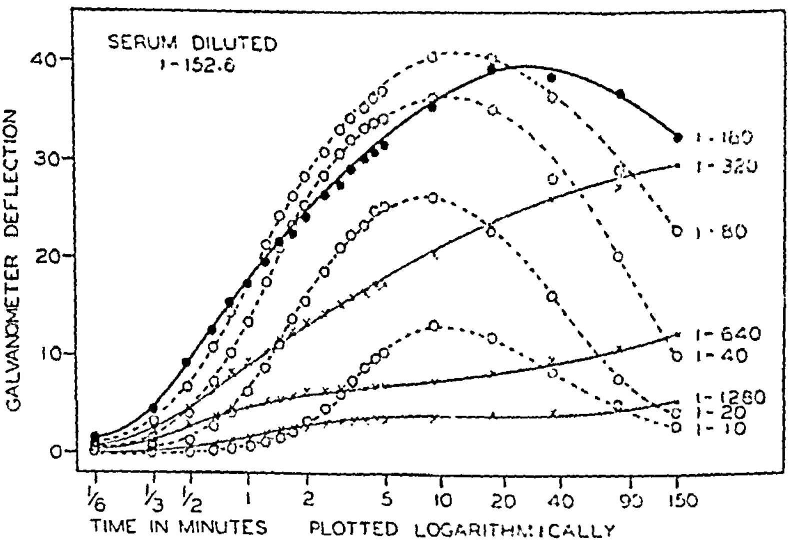


Fig. 1.—Deflection time curves obtained with constant amounts of antibody and various concentrations of antigen over a period of two and one-half hours. Open circles indicate excess antigen was found in the supernatant. Crosses indicate that excess antibody was found in the supernatant. Closed circles indicate that neither antigen nor antibody was found in the supernatant.

The results, plotted in Fig. 1, showed that if observations were continued over a long period of time there was a gradual falling off in galvanometer deflection in the optimal mixture and in mixtures containing excess antigen. Since settling of the precipitate was excluded by shaking of the mixture before placing in the chamber, the fall in deflection resulted from a decrease in the amount of light reflected from the precipitate due either to a reduction in surface-volume ratio or to the absorption of light rays by these very dense suspensions. The precipitates in these mixtures were large and flocculant in contrast to the finely divided turbid suspensions found in all mixtures containing excess of antibody. Although the falling deflection indicated floccule formation, there was no sharp end point and flocculation time could not be estimated. Since none of the mix-

tures showed a decrease in galvanometer deflection during the first five minutes of observation, all subsequent experiments were terminated at this time interval after mixing.

Optimal Proportions in Various Antigen and Antibody Concentrations.—Each of 11 serum dilutions, varying from 1:6.25 to 1:200, was tested with nine concentrations of antigen, varying from 1:5 to 1:1,280 dilutions of the stock solution containing 1 mg. of polysaccharide per milliliter. All dilution figures in the tables and charts are corrected to give the concentration of each reagent per milliliter of mixture.

TABLE I

CORRECTED GALVANOMETER DEFLECTIONS WITH SERUM DILUTED 1:100 AND VARIOUS CONCENTRATIONS OF ANTIGEN

TIME IN SECONDS	ANTIGEN DILUTION								
	1:2,560	1:1,280	1:640	1:320	1:160	1:80	1:40	1:20	1:10
10	0.2	0.3	0.0	0.3	0.5	0.9	0.9	0.8	0.7
20	0.1	0.1	0.1	0.8	2.2	5.1	4.1	2.1	1.0
30	0.1	0.1	0.3	2.2	5.0	11.1	10.0	5.0	1.7
40	0.0	0.1	0.6	3.7	7.9	16.4	16.5	8.7	2.7
50	0.0	0.1	0.8	4.9	10.7	20.4	21.5	13.0	3.8
60	0.1	0.4	1.3	6.1	12.6	23.3	25.9	17.1	5.6
75	0.1	0.5	2.2	7.6	14.9	27.0	30.9	22.7	8.7
90	0.1	0.7	2.7	8.6	16.6	29.2	34.3	26.9	11.5
105	0.1	0.8	3.4	9.4	17.8	31.0	37.0	30.2	14.3
120	0.2	1.0	3.9	9.8	19.0	32.6	39.2	32.9	16.9
150	0.5	1.5	4.7	10.7	20.7	34.9	42.1	36.8	21.1
180	0.8	1.9	5.1	11.6	22.0	36.9	45.0	39.6	24.1
210	0.9	2.1	5.7	12.0	23.1	38.5	46.7	41.2	26.1
240	0.9	2.5	5.8	12.7	23.9	39.7	47.8	42.7	27.4
270	1.0	2.8	6.1	12.9	24.8	40.8	49.0	44.1	28.2
300	1.1	2.9	6.1	13.0	25.3	41.8	50.7	44.8	29.0
Supernatant examination									
Excess antigen	−	−	−	−	−	−	+	+	+
Excess antibody	−	+	−	+	−	−	−	−	−

In Table I are listed the corrected galvanometer deflections obtained with a constant amount of serum (final dilution 1:100) and varying amounts of antigen. The data for all constant antibody series were similar. These data are plotted in Fig. 2 as deflection-time curves to show the progress of the reaction for each antigen dilution. Initially, the most rapid precipitate formation occurred in the mixture containing antigen diluted 1:80 (closed circles), the supernatant of which contained neither antigen nor antibody. With smaller amounts of antigen (region of antibody excess) the precipitate formed more slowly (crosses). The maximal five-minute reading was obtained with antigen diluted 1:40, the supernatant of which was found to contain an excess of antigen (open circles). This dilution of antigen, however, caused a slower initial rate of precipitation than that observed with the optimal antigen dilution. More concentrated antigen resulted in a further slowing of the reaction and a lower galvanometer deflection at the end of the experiment. This was confirmed by all constant antibody series in which the deflections did not exceed the limits of the galvanometer scale during the five-minute period of observation.

The crossings of the time-deflection curves in these experiments illustrate the dangers of interpreting results from a single observation at a single time interval. For example, the same dilution of serum (1:100) will give the same

reading (approximately 20) at the same time interval (approximately one hundred and forty seconds) after mixing with antigen diluted either 1:160 or 1:10. It is obvious that one must know either the shape of the curve or work only within a specified range.

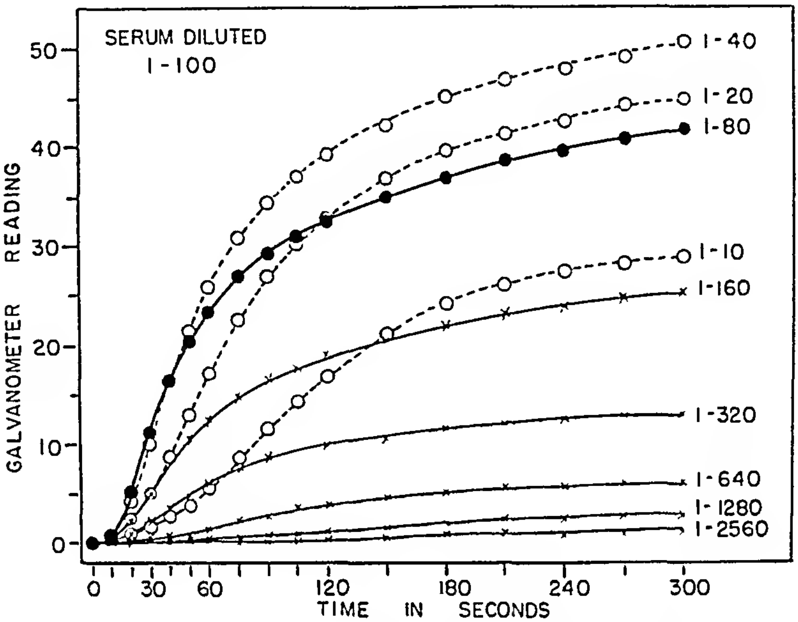


Fig. 2.—Deflection time curves obtained with constant amounts of antibody and varying dilutions of antigen over a 5-minute period. Symbols are the same as in Fig. 1.

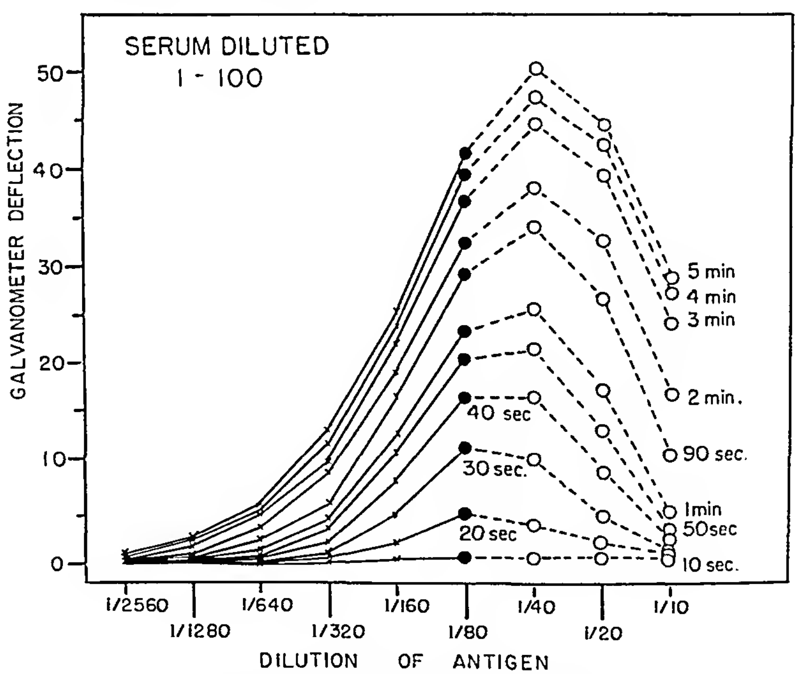


Fig. 3.—Deflection-antigen dilution curves obtained at various time intervals after mixing. Symbols are the same as in Fig. 1.

In Fig. 3 these same data are plotted more conveniently with antigen dilutions as abscissae. This shows again that the 1:80 antigen dilution precipitates fastest during the first thirty seconds. At forty seconds the 1:40 dilution has caught up with the optimal dilution, and after fifty seconds the greatest deflection is obtained with the 1:40 dilution.

TABLE II

CORRECTED GALVANOMETER DEFLECTIONS OF THE LEADING AND ADJACENT TUBES FOR VARIOUS DILUTIONS OF ANTIGEN AND SERUM AT SIGNIFICANT TIME INTERVALS

SERUM DILUTION	TIME IN SECONDS	ANTIGEN DILUTION						
		1:640	1:320	1:160	1:80	1:40	1:20	1:10
1:400	150	0.6	*1.0*	0.5				
1:282.6	40	0.7	*1.0*	0.8	0.6			
1:282.6	240		7.1	*7.2*	4.9			
1:200	40		0.5	*1.0*	0.6	0.5		
1:200	210		6.5	10.8	*11.4*	5.8		
1:141.3	40		0.3	*1.4*	1.1	0.8	0.6	
1:141.3	105		3.5	9.5	*9.9*	9.0	7.0	
1:141.3	210		8.7	17.1	20.6	*20.7*	18.6	
1:100	20			2.2	*5.1*	4.1	2.1	
1:100	40			7.9	16.4	*16.5*	8.7	
1: 70.66	10				2.1	*2.7*	2.0	0.9
1: 70.66	50				27.2	36.0	*36.4*	22.3
1: 50	10					15.0	*17.0*	9.6
1: 35.33	10					32.8	*41.3*	39.6
1: 25	10						57.1	*Off scale*

The deflections of the optimal mixtures are italicized.

The optimal ratios obtained over a fairly wide range of serum dilutions are illustrated by the data presented in Table II. A deflection of 1.0 scale unit was chosen arbitrarily as the smallest value indicating a significant difference in the amount of precipitate. The data in this table were selected to show only the deflections of the optimal and adjacent tubes at the earliest time intervals that significant differences could be measured. The deflection and shifting times of the optimal tube to the mixture containing more antigen are included. These data are plotted in Fig. 4, which shows the results of testing the supernatants for antigen and antibody and indicates the straight line relationship of the optimal tube to both the antigen dilution and the serum dilution. It illustrates also the important point that except in the region of concentrated antibody, the first tube to show definite precipitation lies in the region in which neither antigen nor antibody is present in the supernatant. The maximal five-minute deflection, however, occurs in the region of antigen excess.

Constant Antigen-Varying Antibody Series.—The data, rearranged to compare the reaction speeds obtained with constant amounts of antigen and varying concentrations of antibody, showed that no antibody dilution was optimal for any concentration of antigen. The speed of the reaction increased as the antibody concentration was increased, and in no instance did excess of antibody cause inhibition of the precipitation rate (Fig. 5).

Relationship of Antigen Concentration to Galvanometer Deflection in Region of Serum Excess.—The observations of Bukantz, Cooper, and Bullowa[15] are confirmed by the data presented in Table III, giving the five-minute readings obtained in the regions of serum excess. For antigen dilutions varying from

1:1,280 to 1:160, the galvanometer deflection was directly proportional to the antigen concentration, provided serum was greatly in excess. One unit on the scale represented an average concentration of 0.25 gamma of polysaccharide per milliliter of mixture. These data are plotted in Fig. 6, using the logarithm of the five-minute readings to show the straight line relationship between the log

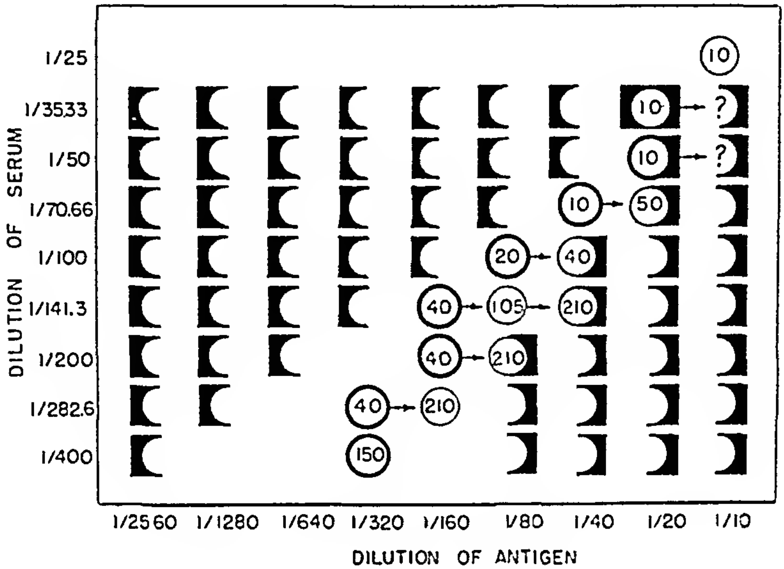


Fig. 4.—Optimal antigen dilutions for varying dilutions of serum. The figure within each circle represents the earliest time interval after mixing at which the deflection of the indicated mixture was significantly higher than deflections obtained at the same time interval with other concentrations of antigen. The results of supernatant examinations are shown by the semicircles, those on the right of the chart indicating excess antigen and those on the left, excess antibody.

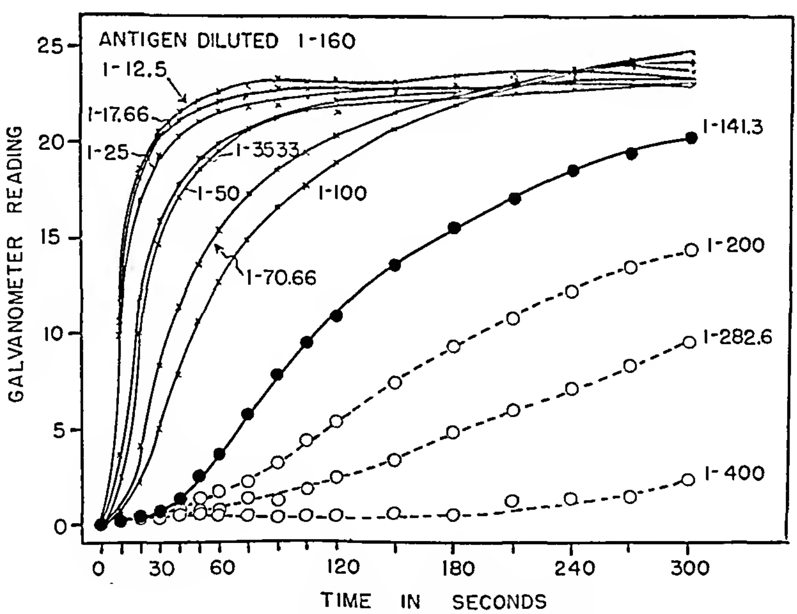


Fig. 5.—Deflection-time curves obtained with constant amounts of antigen and varying amounts of antibody. Symbols are the same as in Fig. 1.

TABLE III

GALVANOMETER DEFLECTIONS FIVE MINUTES AFTER MIXING IN REGION OF EXCESS ANTIBODY

SERUM DILUTION	ANTIGEN DILUTION				
	1:1,280	1:640	1:320	1:160	1:80
1: 12.5	3.5	7.0	13.0	23.2	39.0
1: 17.66	3.1	6.2	12.6	24.0	40.1
1: 25	3.3	7.7	12.7	23.1	41.4
1: 35.33	3.2	6.5	12.6	23.5	41.2
1: 50	3.0	6.8	12.9	24.2	44.9
1: 70.66	2.7	6.2	12.5	24.8	43.6
1:100	2.9	6.1	13.0	25.3	
Mean	3.1	6.6	12.8	24.0	41.7
Weight (in gamma) of polysaccharide causing deflection of one scale division					
	0.25	0.24	0.25	0.26	0.30

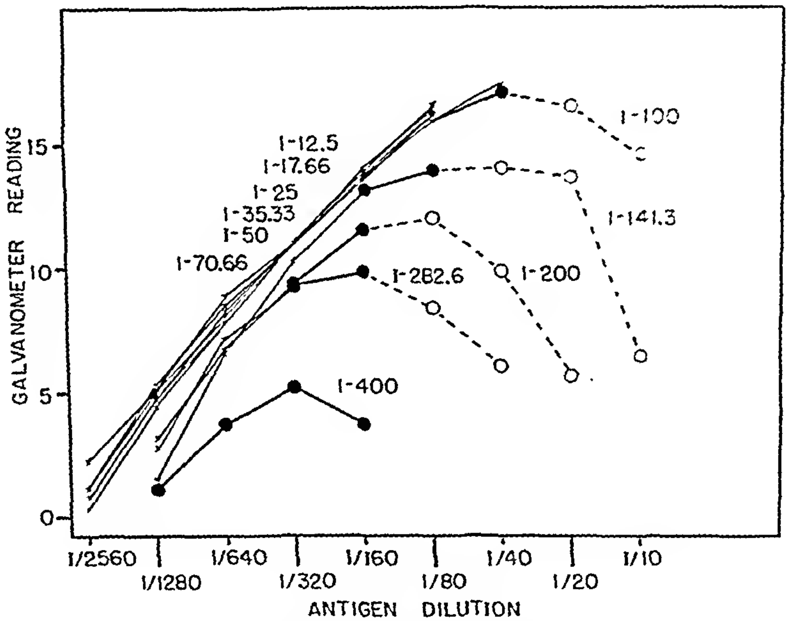


Fig. 6.—Deflection-antigen dilution curves obtained five minutes after mixing, illustrating that in the region of antibody excess there is a straight-line relationship between the galvanometer deflection at constant time and the antigen concentration. Symbols are the same as in Fig. 1.

of the deflection and the log of the antigen concentration. This chart includes also the results obtained in the region of excess antigen (open circles) and the optimal antigen concentration (closed circles). Maximal deflections within five minutes were not attained, as a rule, in the optimal mixtures, but it is possible that these mixtures might have attained the same deflection as those with excess of antibody if observations had been continued for a longer period.

DISCUSSION

No conclusions can be drawn from these experiments concerning the actual rate of reaction between antigen and antibody. The instrument records only the amount of light striking a photoelectric cell after reflection from particles precipitated during the reaction, and there is no evidence that the galvanometer deflection is a function of the weight or the volume of the precipitate. Further-

more, no significance can be attached to the shape of the deflection time curves because the increase in deflection can result from an increase in the number of particles of uniform size, increase in size of a uniform number of particles, or a summation of both factors.

Although the purpose of these experiments was to develop a procedure which could be applied to the problem of precipitin titration in complex antigen-antibody systems, the results indicate that it has certain advantages over other accepted methods of quantitative analysis. Since the optimal proportions principle is valid over a wide range of antigen-antibody dilutions, titration requires only one dilution of serum. With the refined antipneumococcus type I antibody used in these experiments, a complete titration was possible with 14.0 ml. of a 1:100 dilution of serum.

The method is not only more economic of materials but is less time-consuming, a complete run requiring approximately two hours. In comparison with the Dean-Webb procedure the photoelectric method is much more advantageous because it allows time for accurate measurement and thorough mixing of materials. It removes also any doubt concerning the optimal time for reading the reaction, since these experiments have demonstrated that the optimal ratio is determined by noting the fastest reacting mixture as early as possible in the reaction. If the reactions are compared later, the greatest deflection is found in the region of antigen excess.

The optimal ratio, as determined by this method, probably corresponds to the equivalence point described by Heidelberger, since Culbertson[16] showed that the "neutralization" point, as determined by absence of both antigen and antibody in the supernatants, agreed with the results obtained by the chemical method of analysis. A further agreement between the optimal ratio method and the chemical method is evidenced by calculation of the number of molecules of antigen and antibody reacting at the optimal point. Assuming a molecular weight of antibody to be 160,000, the number of molecules in 0.01 ml. of this serum, containing 5.7 mg. of antibody N, or 35.62 mg. antibody protein per milliliter is 13.5 by 10^{14}. The antigen dilution optimal for 0.01 ml. of serum contains 0.0125 mg. SSS per milliliter. Assuming a molecular weight of 5,000, this antigen dilution can be calculated to contain 15.1 by 10^{14} molecules of SSS per milliliter, suggesting that the antigen and antibody molecules combine in a 1:1 ratio when mixed in optimal proportions.

Culbertson[16] emphasized the great inaccuracies of the popular antigen-dilution method of precipitin titration, based upon observing the greatest dilution of antigen causing a visible precipitate with constant amounts of antibody at equilibrium. His contention that such a method was applicable to the titration of antigen content and not to the determination of antibody content is confirmed by these experiments which demonstrate that if excess antibody is present, the galvanometer deflection after a suitable time interval is dependent on antigen concentration alone. Bukantz, Cooper, and Bullowa[15] suggested that the antibody content could be estimated by observing the galvanometer deflection at a constant time interval and noting the concentration of antigen yielding the maximal deflection. However, the maximum deflection in a constant antibody

series is attained always in the region of antigen excess and, therefore, cannot be used as a measure of antibody concentration.

SUMMARY AND CONCLUSIONS

A photoelectric method for determining the relative precipitation rates obtained with various concentrations of antigen and antibody is described. The advantages of this method over the original procedure described by Dean and Webb may be summarized as follows:

1. The various dilutions of antigen and antibody can be measured accurately and mixed thoroughly.

2. The relative precipitation speeds can be determined early in the reaction at the stage which has been shown to be the optimal time for comparing the various precipitation rates.

3. The data are obtained objectively and can be recorded permanently.

From a study of eleven constant antibody-varying antigen series, using pneumococcus type I polysaccharide and antipneumococcus type I rabbit serum, it has been shown that:

1. The fastest initial precipitation occurs in the mixture in which antigen and antibody are in optimal proportions, as shown by absence of both reagents in the supernatant.

2. The maximum galvanometer deflection is obtained in the region of antigen excess.

3. The galvanometer deflection five minutes after mixing is directly proportional to the antigen concentration, provided excess antibody is present.

Comparison of the rates of reaction obtained in nine constant antigen-varying antibody series shows that no dilution of antibody is optimal for a constant amount of antigen. Progressive increases in antibody concentration result in progressively faster precipitation, excess antibody causing no inhibition of the reaction.

The close agreement of Heidelberger's "equivalence point," Culbertson's "neutralization point," and the optimal ratio, as determined by this method, are discussed.

REFERENCES

1. Dean, H. R., and Webb, R. A.: The Influence of Optimal Proportions of Antigen and Antibody in the Serum Precipitation Reaction, J. Path. & Bact. 29: 473, 1926.
2. Heidelberger, M.: Quantitative Absolute Methods in the Study of Antigen-Antibody Reactions, Bact. Rev. 3: 49, 1939.
3. Heidelberger, M., and Kendall, F. E.: A Quantitative Study of the Precipitin Reaction Between Type III Pneumococcus Polysaccharide and Purified Homologous Antibody, J. Exper. Med. 50: 809, 1929.
4. Heidelberger, M., and Kabat, E. A.: Chemical Studies on Bacterial Agglutination, J. Exper. Med. 60: 643, 1934.
5. Ramon, G.: Flocculation dans un melange neutre de toxine-antitoxine Diphtheriques, Compt. rend. Soc. de biol. 86: 661, 1922.
6. Marrack, J. R.: The Chemistry of Antigens and Antibodies, Medical Research Council, Special Report Series, No. 194, 1934.
7. Brown, A. M.: Optimal Precipitin Reactions, Brit. J. Exper. Path. 16: 554, 1935.
8. Taylor, G. L.: The Results of Some Quantitative Experiments on the Serum Precipitation Reaction, J. Hyg. 31: 56, 1931.
9. Taylor, G. L.: The Dissimilarity of the Results of Precipitin Titrations Performed With a Constant Amount of Antiserum and With a Constant Amount of Antigen, J. Hyg. 33: 12, 1933.

10. Dean, H. R.: The Relation Between the Fixation of Complement and the Formation of a Precipitate, Ztschr. f. Immunitätsforsch. u. exper. Therap. 13: 84, 1912.
11. Smith, W.: The Titration of Antipneumococcus Serum, J. Path. & Bact. 35: 509, 1932.
12. Libby, R. L.: The Photronreflectometer—An Instrument for the Measurement of Turbid Systems, J. Immunol. 34: 71, 1938.
13. Libby, R. L.: A New and Rapid Quantitative Technique for the Determination of the Potency of Types I and II Antipneumococcal Serum, J. Immunol. 34: 269, 1938.
14. Libby, R. L.: A Simplified Photronreflectometric Technic for the Titration of Antipneumococcal Horse and Rabbit Serum, J. Immunol. 35: 289, 1938.
15. Bukantz, S. C., Cooper, A., and Bullowa, J. G. M.: The Photoelectric Titration of Pneumococcal Capsular Polysaccharide, J. Bact. 42: 15, 1941.
16. Culbertson, J. T.: A Quantitative Study of the Precipitin Reaction With Special Reference to Crystalline Egg Albumin and Its Antibody, J. Immunol. 23: 439, 1932.

THE KAHN VERIFICATION TEST IN MALARIA

ALBERT DE GROAT,* CAMP RUCKER, ALA.

KAHN has recently published a verification test[1] for the detection of false positive reactions in the serology of syphilis. More recently, Kahn, McDermott, and Marcus[2] and Jacobsthal[3] have reported that this test is capable of differentiating positive serologic reactions in syphilis from those occurring in malaria. An opportunity was afforded me to apply the verification test to a group of clinically nonsyphilitic soldiers with malaria who gave positive serologic reactions for syphilis. This article will present the results obtained.

The verification test is based on the principle that syphilitic serums giving positive serologic reactions show under proper conditions more marked precipitation at 37° than at 1° C. (syphilitic type of reaction), while nonsyphilitic serums giving positive reactions show under the same conditions more marked precipitation at 1° than at 37° C. (general bio-

TABLE I

SEROLOGIC RECORD OF MALARIAL CASES

NAME	DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
		STANDARD TEST	PRESUMPTIVE TEST		
J. Z.	10/24/41	Pos. (++++)	Pos. (++++)	200	General biologic
N. A.	11/ 3/41	Neg.	Neg.	0	General biologic
M. T.	11/ 3/41	Neg.	Neg.	0	Negative
M. J.	11/ 3/41	Neg.	Neg.	0	General biologic
T. F.	11/ 3/41	Neg.	Pos. (+++)	0	General biologic
E. H.	11/ 3/41	Neg.	Neg.	0	General biologic
W. C.	11/ 3/41	Pos. (+++)	Pos. (++++)	3	General biologic
M. K.	11/ 3/41	Neg.	Neg.	0	Negative
H. C.	11/ 3/41	Pos. (++++)	Pos. (++++)	80	General biologic
A. L.	11/ 3/41	Neg.	Neg. (+)	0	General biologic
R. W.*	11/ 3/41	Neg.	Neg.	0	General biologic
W. J.	11/ 3/41	Neg.	Neg.	0	Negative
C. A.	11/ 3/41	Neg.	Pos. (+++)	0	General biologic
J. Q.	11/ 3/41	Pos. (++)	Pos. (++++)	2	General biologic
R. M.	11/ 3/41	Doubt. (+)	Pos. (++++)	1	General biologic
D. L.*	11/ 7/41	Pos. (++++)	Pos. (++++)	40	General biologic
E. G.	11/ 7/41	Pos. (++++)	Pos. (++++)	40	Inconclusive
A. D.	11/14/41	Pos. (++++)	Pos. (++++)	4	General biologic

*Nonmalarial cases: see Table IV.

*Major, Medical Corps, U. S. Army.
From the Station Hospital Laboratory.
Received for publication, June 25, 1942.

logic, nonsyphilitic, type of reaction). Serums giving negative serologic reactions generally show no precipitation at 37° or 1° C., and the verification reaction is referred to as negative type. When precipitation of equal strength is obtained at these two temperatures, the verification results are referred to as inconclusive.

Blood specimens from the seropositive cases of malaria and also from some seronegative cases were sent to Doctor Kahn's laboratory at the University of Michigan Hospital for verification tests. No indications as to the source of these specimens were submitted. The serologic reports received from that laboratory are summarized in Table I. Of eighteen serums examined, the standard Kahn test proved to be positive in seven, doubtful in one, and negative in the remaining ten. The supersensitive presumptive test gave ten positive reactions in the same group of serums. All these positive reactions, according to the verification test, were of the general biologic (nonsyphilitic) type, except in one instance in which the result of the verification test was reported as inconclusive.

TABLE II

KAHN VERIFICATION TEST IN SEROPOSITIVE CASES OF MALARIA

DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
	STANDARD TEST	PRESUMPTIVE TEST		
		Case 1 (J. Z.)		
10/24/41	Pos. (+ + + +)	Pos. (+ + + -)	200	General biologic
11/ 3/41	Pos. (+ + + +)	Pos. (+ + + +)	80	General biologic
11/ 7/41	Pos. (+ + +)	Pos. (+ + + +)	3	General biologic
11/15/41	Neg.	Neg.	0	General biologic
		Case 2 (H. C.)		
11/ 3/41	Pos. (+ + + +)	Pos. (+ + + +)	80	General biologic
11/ 7/41	Pos. (+ + +)	Pos. (+ + + +)	3	General biologic
12/ 6/41	Neg.	Neg.	0	General biologic
12/31/41	Neg.	Neg.	0	Negative
		Case 3 (W. C.)		
11/ 3/41	Pos. (+ + +)	Pos. (+ + + +)	3	General biologic
11/ 7/41	Neg.	Pos. (+ + +)	0	General biologic
11/14/41	Neg.	Neg.	0	General biologic
12/31/41	Neg.	Neg.	0	Negative
		Case 4 (J. Q.)		
11/ 3/41	Pos. (+ +)	Pos. (+ + + +)	2	General biologic
11/ 7/41	Pos. (+ +)	Pos. (+ + + -)	2	General biologic
11/27/41	Neg.	Neg.	0	Negative
12/ 4/41	Neg.	Neg.	0	Negative
		Case 5 (R. M.)		
11/ 3/41	Doubt. (-)	Pos. (+ + - -)	1	General biologic
11/ 7/41	Neg.	Doubt. (+ +)	0	General biologic
12/ 6/41	Neg.	Neg.	0	Negative
		Case 6 (E. G.)		
11/ 7/41	Pos. (+ + - +)	Pos. (+ + + +)	40	Inconclusive
11/14/41	Neg.	Pos. (- + + +)	0	General biologic
12/ 4/41	Neg.	Neg.	0	Negative
12/11/41	Neg.	Neg.	0	Negative
		Case 7 (A. D.)		
11/14/41	Pos. (+ + - -)	Pos. (+ + + -)	4	General biologic
11/25/41	Pos. (- + +)	Pos. (+ - + -)	3	General biologic
1/30/42	Neg.	Neg.	0	Negative

Of interest is the fact that five of the negative reactions with the standard and presumptive Kahn tests were also, according to the verification test, of the general biologic (nonsyphilitic) type. Generally, serums that give negative reactions with serodiagnostic tests give the negative type of reaction with the verification test. The general biologic type of reaction given by these negative reacting serums indicates, according to Kahn, that these serums contain nonsyphilitic "antibody" and that they might give positive reactions with certain serodiagnostic tests and, under certain conditions, with perhaps all tests.

Of interest is the fact also that, according to Table I, malarial serums may give high titers with the quantitative Kahn reaction. Two of the serums gave a titer of 40, one of 80, and one of 200. Evidently positive reactions in malaria are not limited to low serologic titers.

The next problem that presented itself was to find if the general biologic (nonsyphilitic) type of reactions obtained in the patients with malaria could be accepted as serologic evidence of the absence of syphilis in these cases. Repeated serologic examinations of the blood specimens in these cases, it was believed, would throw light on this problem. Thus, positive serologic reactions in acute malaria generally become negative after recovery. Would the positive reactions in the patients under investigation persist or would they become

Table III

Kahn Verification Test in Cases of Malaria, Essentially Seronegative

Date	Kahn Reactions		Quantitative Serologic Units	Verification Test (Type of Reaction)
	Standard Test	Presumptive Test		
		Case 8 (T. F.)		
11/ 3/41	Neg.	Pos. (+ + +)	0	General biologic
11/ 7/41	Neg.	Neg.	0	General biologic
11/14/41	Neg.	Neg.	0	General biologic
11/21/41	Neg.	Pos. (+ + + +)	0	General biologic
11/29/41	Neg.	Neg.	0	General biologic
1/16/42	Neg.	Neg.	0	Negative
		Case 9 (C. A.)		
11/ 3/41	Neg.	Pos. (+ + +)	0	General biologic
11/14/41	Neg.	Neg. (+)	0	General biologic
11/21/41	Neg.	Neg.	0	General biologic
11/28/41	Neg.	Neg.	0	General biologic
12/ 4/41	Neg.	Neg.	0	General biologic
12/11/41	Neg.	Neg.	0	Negative
		Case 10 (A. L.)		
11/ 3/41	Neg.	Neg. (+)	0	General biologic
11/ 7/41	Neg.	Neg. (+)	0	General biologic
11/25/41	Neg.	Neg.	0	General biologic
1/ 7/42	Neg.	Neg.	0	General biologic
		Case 11 (E. H.)		
11/ 3/41	Neg.	Neg.	0	General biologic
11/ 7/41	Pos. (+ +)	Pos. (+ + + +)	2	General biologic
11/15/41	Neg.	Neg.	0	General biologic
11/25/41	Neg.	Neg.	0	Negative
12/ 2/41	Neg.	Neg.	0	Negative
		Case 12 (M. J.)		
11/ 3/41	Neg.	Neg.	0	General biologic
11/21/41	Neg.	Neg.	0	General biologic
12/ 4/41	Neg.	Neg.	0	Negative

negative with the subsidence of the fever and with recovery? Accordingly, arrangements were made for the serologic follow-up of these patients.

Table II lists seven seropositive cases of malaria which became seronegative in about one to three months. The verification reactions in these patients changed from the general biologic type to the negative type of reactions. An exception is Case 1 in which the original general biologic type of reaction persisted. Table III lists five cases of malaria, essentially seronegative, except that with the supersensitive presumptive test, seven positive reactions of varying degree were obtained through the period of investigation. In these cases also, the verification test at first gave the general biologic type of reactions, and later, the negative type.

Two febrile patients of the original group of twenty-one, listed in Table IV, were found not to have malaria. One patient with cellulitis (Case 13) was Kahn-negative. Another (Case 14), with an acute respiratory infection, was Kahn-positive. The verification test, in both cases, gave the general biologic type of reactions. On repeated serologic examinations, the Kahn-positive reaction rapidly became negative. But in both cases, the general biologic type of verification reactions persisted, although the patients had recovered. It was impossible to continue verification studies in these patients due to the transfer of the soldiers from Fort Custer.

TABLE IV

KAHN VERIFICATION TEST IN TWO NONMALARIAL CASES

DATE	KAHN REACTIONS		QUANTITATIVE SEROLOGIC UNITS	VERIFICATION TEST (TYPE OF REACTION)
	STANDARD TEST	PRESUMPTIVE TEST		
Case 13 (R. W.); Cellulitis, Chills, and Fever				
11/ 3/41	Neg.	Neg.	0	General biologic
12/ 4/41	Neg.	Neg.	0	General biologic
1/ 7/42	Neg.	Neg.	0	General biologic
Case 14 (D. L.); Acute Respiratory Infection				
11/ 7/41	Pos. (+ + + +)	Pos. (+ + + +)	40	General biologic
11/14/41	Neg.	Neg. (+)	0	General biologic
11/21/41	Neg.	Neg.	0	General biologic

SUMMARY

The Kahn verification test was applied to a group of clinically nonsyphilitic soldiers with malaria giving positive serologic reactions for syphilis. It was found that these soldiers gave the general biologic (nonsyphilitic) type of reactions, indicating that the verification test can be employed as an aid in establishing the differential diagnosis of malaria and syphilis.

REFERENCES

1. Kahn, R. L.: Serologic Verification Test in Diagnosis of Latent Syphilis, Arch. Dermat. & Syph. 41: 817, 1940.
2. Kahn, R. L., McDermott, E. B., and Marcus, S.: Effect of Temperature of Kahn Reaction With Serologically Positive Sera of Lower Animals, Amer. J. Syph., Gonor. & Ven. Dis. 25: 151, 1941.
3. Jacobsthal, E.: Sobre el Princípio del Verification Test, Bol. Sanit. de Guatemala 12: 190, 1941.

DETERMINATION OF BLOOD PRESSURE IN RATS BY DIRECT OBSERVATION OF BLOOD VESSELS*

George W. Duncan, M.D., Baltimore, Md., and Chester Hyman, M.S., and Edward L. Chambers, B.A., New York, N. Y.

Although several methods of determining blood pressure in small animals have been described, certain complicating features in the technique of each of them often render them inapplicable to the experimental problem on hand. It is the purpose of this communication to point out briefly the essential features of previously described methods and to present a simple method for repeated determinations which produces no trauma or loss of blood and which may be used on either anesthetized or unanesthetized animals.

Methods involving direct cannulation of large vessels require either local or general anesthesia. Trauma and loss of blood result to a greater or lesser degree during exposure and cannulation of the vessel, and in most of the methods of this type the animal is usually sacrificed. The method of Woodbury and Hamilton[1] has definite advantages in some problems but is not simple technically, the apparatus is expensive, and each determination requires surgical exposure of the vessels to be punctured. The values obtained by these investigators are higher than pressures obtained, also by arterial cannulation, by Rous and Drury[2] and by Chanutin and Ferris.[3] Diaz and Levy[4] described a method for determining blood pressure in rats which depends upon the flow of blood from the incised tail when the pressure in the arteries exceeds that in a pneumatic cuff about the base of the animal's tail. The values obtained by these authors coincided rather closely with pressures obtained by aortic cannulation. This method produces loss of blood and trauma with each determination, and where frequent determinations are made, this degree of blood loss may complicate certain types of experiments.

Indirect methods which necessitate no trauma or loss of blood have been described by several authors. Kunstmann[5] and Bonsmann[6] made use of the restoration of pink color due to the return of blood to a limb or tail which has been previously emptied of blood by a tight bandage. The flow of blood is controlled by means of a compression cuff at the base of the animal's tail. The values obtained by both these investigators are lower than the values reported by other investigators. Williams, Harrison, and Grollman[7] have described a practical and accurate method which is based on plethysmographic measurement of the increase in volume of the rat's tail when the pressure in the arteries exceeds that in a compression cuff about the animal's tail. This method is used on trained rats and requires preliminary warming of the animal for accuracy. A similar method was described by Byrom and Wilson.[8]

*From the Department of Surgery, The Johns Hopkins University, and Department of Biology, Washington Square College, New York University.

Received for publication, Sept. 11, 1942.

Two methods have been described which involve the application of a compression cuff to the thigh and observation of the flow of blood in the small blood vessels of the foot. Griffith[9] observed microscopically the cessation and restoration of flow in the small superficial vessels on the dorsum of the rat's foot. The observations were made with strong reflected light on an area that had been depilated and cleared with immersion oil. He recommended that several fields be observed because of the uncertainty of the type of vessels under observation. We were unable to obtain consistent results by this method. McMaster[10] reported a similar method in which flow in the blood vessels at the base of the claws of mice was observed by transmitted light. He pointed out that similar observations cannot be made in rats, even in young ones.

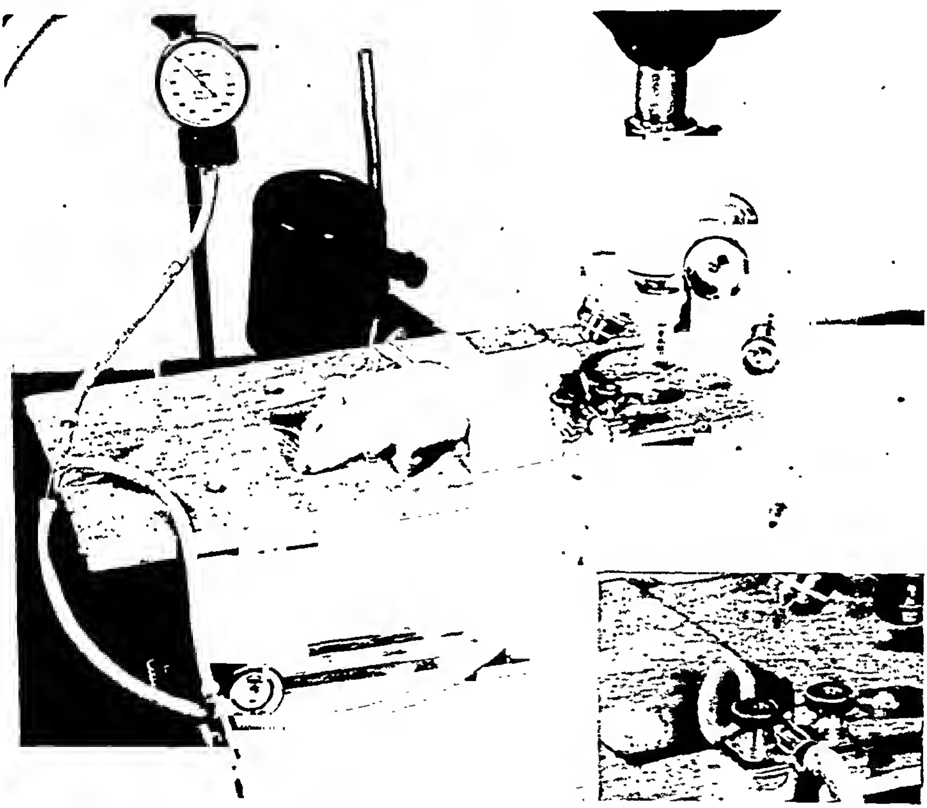


Fig. 1.—Arrangement of apparatus for determining blood pressure of rat. Insert shows details of pressure cuff and transillumination cone.

The method to be described is also based upon direct observation of blood vessels. The vessels are those of the translucent interdigital web and are observed by means of transmitted light. These vessels of the spread-out web are clearly visible so that the different types of vessels can be easily identified.

Normal rats weighing from 130 to 200 Gm. were used. The apparatus, shown in Fig. 1, consists of a cuff, similar to that described by Diaz and Levy[4] and Byrom and Wilson,[5] which is placed about the thigh. The cuff is made of a 2 cm. length of glass tubing 2 cm. in diameter and flanged on both ends, with a short length of 4 mm. glass tubing fused to the side for connection with a manometer and a rubber pressure bulb. A piece of ⅝ inch Penrose tubing

is placed inside the larger glass tube, everted over the flanged ends, and cemented to the outer surface. The cuff is attached to a board 5 by 12 inches in such a position that it rests about four inches from the end. The unanesthetized rat is kept fairly quiet by placing it in a snugly fitting glass cylinder on the end of the board carrying the cuff. One hind limb is drawn through the cuff to the level of the upper thigh and held in place by small clamps attached to the claws or by strings tied about the distal ends of two adjacent digits. The position of the foot is such that the spread-out interdigital web rests upon the upper surface of truncated glass cone fixed through the board. The cone measures approximately 1 cm. at its base, and its upper end is ground to a triangular cross section which measures approximately 0.25 cm. on each side and fits closely between the outspread digits. The web is rendered translucent by removal of the epidermal scurf by a brief application of depilatory cream and clearing with almond oil or paraffin oil. A small piece of cover slip placed upon the web serves to hold the film of oil in place and thus an even surface for observation is presented. The board with the animal in place is mounted on the stage of the microscope so that the truncated cone of glass lies in the optical axis. Light is obtained from a Leitz low voltage microscope lamp or any equivalent source. With a magnification of 100 times the vessels and the flow of blood can be clearly seen. The small arterial vessels can be differentiated from corresponding veins by their relatively thicker walls and more rapid rate of flow and by the direction of flow.

In making a determination the cuff is inflated to a pressure sufficient to stop all flow of blood. The pressure is then slowly lowered until the first continuous flow becomes visible in the arteriole under observation. The pressure at that time is taken as the blood pressure in the large arteries of the thigh. Other vessels in the field are checked to ensure accuracy.

The normal blood pressures of different animals of the unanesthetized group ranged from 90 to 140 mm. Hg. The range of pressures in different animals of the anesthetized group was from 70 to 120 mm. Hg. Repeated determinations were made on several animals of each group and were found to agree within 4 mm. Hg in any given animal. The accuracy of the method was checked by obtaining pressures by aortic cannulation on six rats immediately following determinations by the interdigital web method. The values checked within 5 mm. Hg.

SUMMARY

The arterial blood pressure of rats may be rapidly and easily determined by microscopic observation of the flow of blood in the small arterial vessels of the interdigital web. Values obtained by this method agree with pressures determined by aortic cannulation and with those reported in the literature.

REFERENCES

1. Woodbury, R. A., and Hamilton, W. F.: Blood Pressure Studies in Small Animals, Am. J. Physiol. 119: 663, 1937.
2. Rous, P., and Drury, D. R.: Outlying Acidosis Due to Functional Ischemia, J. Exper. Med. 49: 435, 1929.
3. Chanutin, A., and Ferris, E. B.: Experimental Renal Insufficiency Produced by Partial Nephrectomy; Control Diet, Arch. Int. Med. 49: 767, 1932.

4. Diaz, J. T., and Levy, S. E.: An Indirect Method for Repeated Determinations of Blood Pressure of Rats, Proc. Soc. Exper. Biol. & Med. 40: 402, 1939.
5. Kunstmann, H. K.: Ein Verfahren zur Blutdruckbestimmung bei Maus, Ratte und Kaninchen, Arch. f. exper. Path. u. Pharmakol. 132: 122, 1928.
6. Bonsmann, M. R.: Blutdruckversuche an der Maus und Ratte mittels Photozelle, Arch. f. exper. Path. u. Pharmakol. 176: 460, 1934.
7. Williams, J., Harrison, T. R., and Grollman, A.: A Simple Method for Determining the Systolic Blood Pressure of the Unanesthetized Rat, J. Clin. Investigation 18: 373, 1939.
8. Byrom, F. B., and Wilson, C.: A Plethysmographic Method for Measuring Systolic Blood Pressure in the Intact Rat, J. Physiol. 93: 301, 1938.
9. Griffith, J. Q., Jr.: Indirect Method for Determining Blood Pressure in Small Animals, Proc. Soc. Exper. Biol. & Med. 32: 394, 1934.
10. McMaster, P. D.: A Method to Determine Peripheral Arterial Blood Pressure in the Mouse, J. Exper. Med. 74: 29, 1941.

A METHOD FOR OBTAINING RECORDS OF THE TORTOISE SINUS WITH DATA ON EXTRASYSTOLES AND LENGTH OF CYCLES FOLLOWING STIMULATION

FREDERICK E. EMERY, PH.D., AND T. A. LOOMIS, M.S., BUFFALO, N. Y.

During the last several years graphic records taken from the beating sinus venosus of the tortoise have been used in discussing with medical students the refractory period, extra systole, and compensatory pause of the heart. These phenomena in the ventricles and to some extent in the auricles are common descriptions in most textbooks, and a discussion of excitations near the great veins or sinoauricular node is nicely presented by Wiggers.[1] Graphic records taken from the sinus are not shown in any of the present textbooks, nor have we found them in any of the literature so far reviewed. It seems worth consideration, therefore, to describe briefly the method used to obtain records of the sinus venosus and to exhibit some of the records obtained. The literature is, for the most part, of long standing, and excellent reviews are available.[2, 3] These and original contributions having a direct bearing on the problem are discussed briefly as their citation appears in the body of this paper.

PROCEDURE

Twenty-seven pithed tortoises about six inches in shell length were used, and from these about two thousand records of sinus stimulation were saved for study. The usual method of recording the auricular and ventricular contraction by means of aluminum heart levers was employed. In order to obtain tracings of the beating sinus, fine thread was usually attached directly to the sinus (5 to 8 mm. to the right of the midline) with the aid of fine-pointed dissecting forceps. In a few cases the thread was placed around the large veins to the right of the sinus. The heart lever required careful counter balance and adjustment, but when so arranged usually gave a clear tracing of the sinus beats. By means of a small, bent glass rod with a hook at the end the base of the ventricle could be adjusted to the left and ventrally in such a way

Department of Physiology, University of Buffalo.
Received for publication, July 6, 1942.

as to free the sinus tracing from auricular and ventricular interference. One stimulating electrode of fine copper wire was placed on and sometimes directly into the wall of the sinus, and the second electrode was stuck in the liver one inch back from the heart and on the midline. Make-and-break stimuli from a Harvard inductorium were given. Best results were obtained if the heart rate was slow, so in six experiments the rates were slowed by placing the shell on ice during the entire experiment. A depression in a large cake of ice was chipped out to fit the contour of the shell and, when the tortoise was placed in this, satisfactory slowing of the heart rate soon followed.

RESULTS

Tracings taken simultaneously from the sinus, auricles, and ventricles show the normal sequence of activity in these respective parts as the contracting wave moves over the heart.

Fig. 1*B* shows that stimulation of the sinus in the middle third of the cycle with a relatively weak stimulus (make) has no effect on the rhythm, while the break stimulus applied at the same time of the cycle produced an extra con-

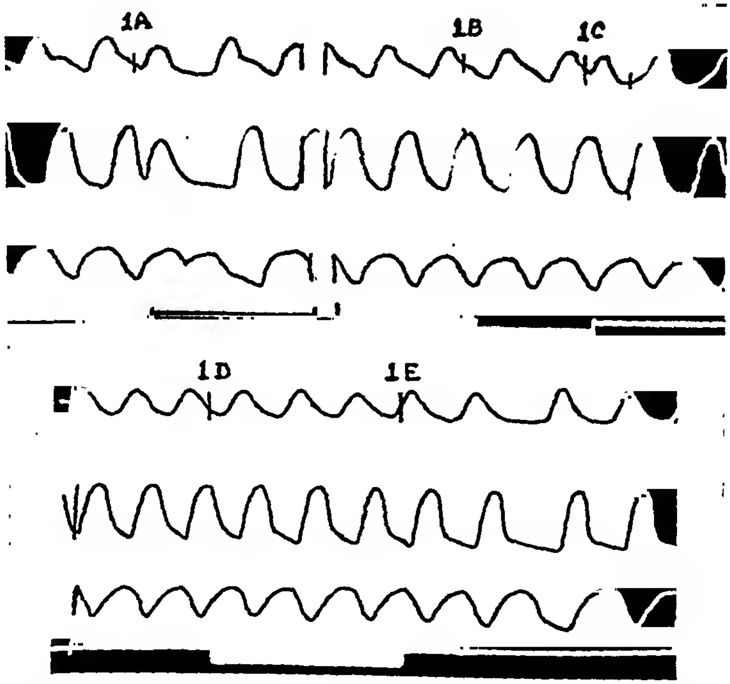


Fig. 1.—Heart rate, 17 per minute. Tracings from above downward; sinus, auricles, ventricles, signal magnet (down stroke make stimulus, up stroke break stimulus). Arrows show where the stimulus was applied to the sinus (one dry cell, Harvard coil, 4 cm.).

A, A stimulus applied to the sinus in the last third of diastole induced an extrasystole. The pause following is longer than normal. The "postcompensatory" contraction of the sinus is higher than normal.

B, A make stimulus, just below threshold for this tortoise, applied to the sinus in the middle third of the cycle had no effect.

C, The break stimulus applied in the same phase of the cycle as in *B* was followed by an extrasystole and a pause of shorter duration than normal. The "postcompensatory" contraction of the sinus is slightly higher than normal.

D, A make (in this case subthreshold) stimulus applied in the last third of the cycle had no effect.

E, Since the make stimulus as in *D* was subthreshold, the break stimulus as applied was relatively weak, yet the disturbance induced by stimulation in the refractory period caused the next three pauses of the sinus to be longer.

traction in the auricles and ventricles (Fig. 1C). In this case the extra contraction of the sinus was followed after a pause of shorter duration than normal by the usual rhythmic sinus discharges. Lewis[3] describes sinus extrasystoles as rare in the human subject but points out that the returning cycle may be shorter than normal. Engelmann[4] showed in amphibians that an extrasystole induced by stimulating the sinus was followed by returning cycles of the same length as the initial cycles. In our experience if the stimulus is applied to the sinus in the middle half of the cycle, the pause following the extrasystole may be of normal length about 20 per cent of the time or, using the expression of

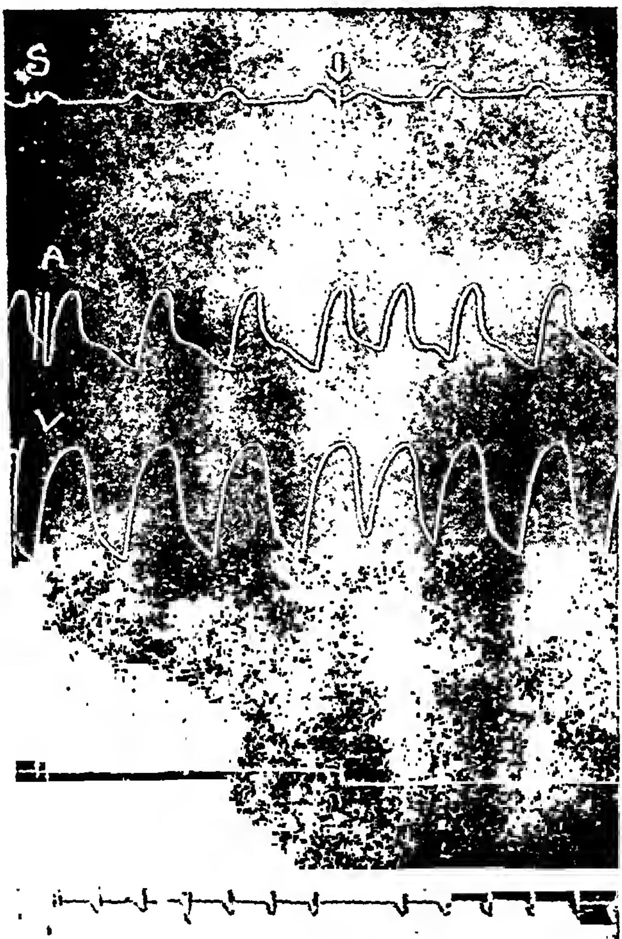


Fig. 2.—Time in 5 seconds, otherwise tracings and coil same as in Fig. 1. A stimulus applied at the beginning of the middle third of the cycle induced an extrasystole of the sinus which was carried to the auricles and ventricles. This induced cycle was approximately equal in length to the preceding cycle.

Lewis, the returning cycle is approximately equal in length to the initial cycles (Fig. 2). On the other hand, the next discharge of the sinus, after the extrasystole, may be delayed as compared to initial cycles or subsequent returning cycles (Figs. 1A, 3 A and B, 4B). Cushny,[5] working with the isolated ventricle of mammals, introduced the idea of fatigue of the pacemaker due to extra impulses either from artificial stimuli or "impulses reaching it from the

Keith-Flack node.'' Lewis[3] has discussed this point in regard to the sinoauricular node and states that ''The manner and circumstances in which this depressant effect of an extrasystole is exerted are not yet entirely clear.''

In order to study factors which might determine the length of the pause following an extrasystole of the sinus, 772 records were carefully measured with the aid of compass and lens. This study revealed that a stimulus applied to the sinus during contraction (refractory period) always failed to cause an

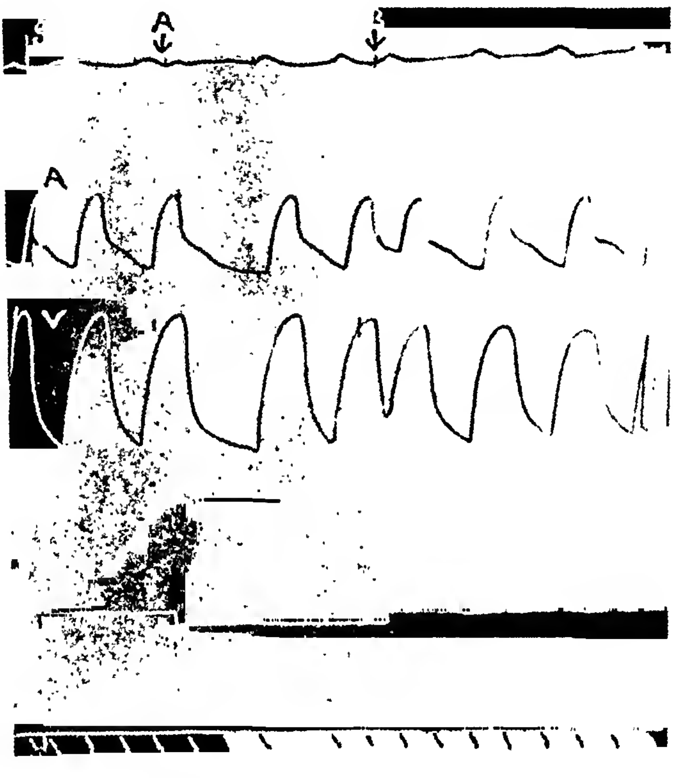


Fig. 3.—Time in 5 seconds, otherwise tracings and coil same as in Fig. 1.

A, Stimulus applied to the sinus at the beginning of the middle third of the cycle. Extra contraction of the sinus not carried to the auricles and ventricles. "Postcompensatory" contraction of auricles and ventricles higher than normal. Induced cycle slightly longer than the preceding cycle.

B, Stimulus applied in the middle third of the cycle. Extrasystole of the sinus carried to auricles and ventricles, followed by a pause of longer duration than normal.

extra contraction but the rhythm may be disturbed. In about one-fourth of the records the next pause was longer (Table I), and sometimes this disturbance was evident in the next two or more following cycles (Fig. 1*E*). A stimulus of at least threshold strength for the sinus but applied during the relaxation of the sinus (descending limb of the record) often induced a change in rhythm, and the subsequent pause was altered, being shorter in a few records (3.4 per cent), normal in 53.0 per cent, and longer in 43.6 per cent (Table I). This period may be looked upon as the relative refractory period and variable in length as subsequent discussion will show.

A stimulus applied in the first quarter of the cycle usually gave no extrasystole, but in a few cases where an extrasystole occurred the pause that

followed was always of shorter duration than normal. Table I shows a summary of nineteen records of tortoises in which this type of response occurred. A stimulus falling within the first third of the cycle gave an extrasystole which was followed in 77.1 per cent by a pause of shorter duration than normal and in 9.4 per cent by a longer pause than normal (Table I). A stimulus applied during the middle third of the cycle gave an extrasystole and a pause which was shorter than normal in 54.1 per cent. In contrast to these examples, a stimulus given in the last third of the cycle caused as expected an extrasystole, but the pause that followed was usually (80.0 per cent) longer than normal (Table I).

TABLE I

The length of 772 cycles following stimulation is compared to the previous cycle. In records of tortoises in which the stimulus was not applied in the refractory period or relative refractory period, the following cycles included an extrasystole in each case.

STIMULATED IN:	NO. OF RECORDS	LENGTH OF NEXT CYCLE FOLLOWING EXTRASYSTOLES OR STIMULATION		
		SHORTER	SAME	LONGER
Refractory period	100	0	77	23
Relative refractory period	87	3 (3.4%)	46 (53.0%)	38 (43.6%)
First quarter of cycle	19	19	0	0
First third of cycle	96	74 (77.1%)	13 (13.5%)	9 (9.4%)
Middle third of cycle	270	146 (54.1%)	56 (20.7%)	68 (25.2%)
Last third of cycle	200	17 (8.5%)	23 (11.5%)	160 (80.0%)

Thus it is clear that the length of the pause that follows an extrasystole in the sinus of the tortoise is not fixed but closely related to the time in the cycle when the stimulus was applied (Figs. 1 to 4). The earlier in the cycle that an effective stimulus is applied, the shorter the pause is likely to be, and conversely a stimulus applied in late diastole of the sinus is almost certain to be followed by a pause of longer duration than normal. At least two factors have been found that tend to change these expected results. The first of these deals with the relative refractory period of the sinus which, in our experience, varied in length in different tortoises. When this period is long, it is most difficult to obtain an extrasystole sufficiently early in the cycle to give the expected pause of shorter duration. Fig. 4*A* illustrates this very nicely and shows that a make stimulus applied in the middle third of the cycle had no effect. After a pause of a few seconds a second record was taken, and the same strength of stimulus applied slightly later in the cycle induced an extrasystole which was followed by a pause of longer duration than normal (Fig. 4*B*). In other words, it was impossible to get a reaction in the first third of the cycle with a stimulus which was considerably above threshold. The second factor also tends to fix the length of the pause but operates when the stimulus is applied in late diastole of the sinus. In these records the induced or extrasystole occurred at the expected time of the normal contraction and was followed by a pause of normal length.

The disturbance in the sinus induced by stimulation may be followed by a delay of the next rhythmic discharge, even though the artificial stimulus may have been so weak that the sinus contracted only slightly (Fig. 3*A*). This record also shows that the stimulus was either too weak to be effectively transmitted to the auricles or the auricles had not recovered sufficiently to respond.

the result being a pause in auricular and ventricular activity until the next sinus discharge, which was itself delayed. The strength of the stimulus has some effect on the length of the pause that follows an extrasystole. If the stimulus is very strong, the pause is likely to be modified in the direction of longer length. However, careful check of many records shows that the strength of the stimulus within reasonable limits does not alter the expected results in regard to the length of the pause that follows the extra contraction.

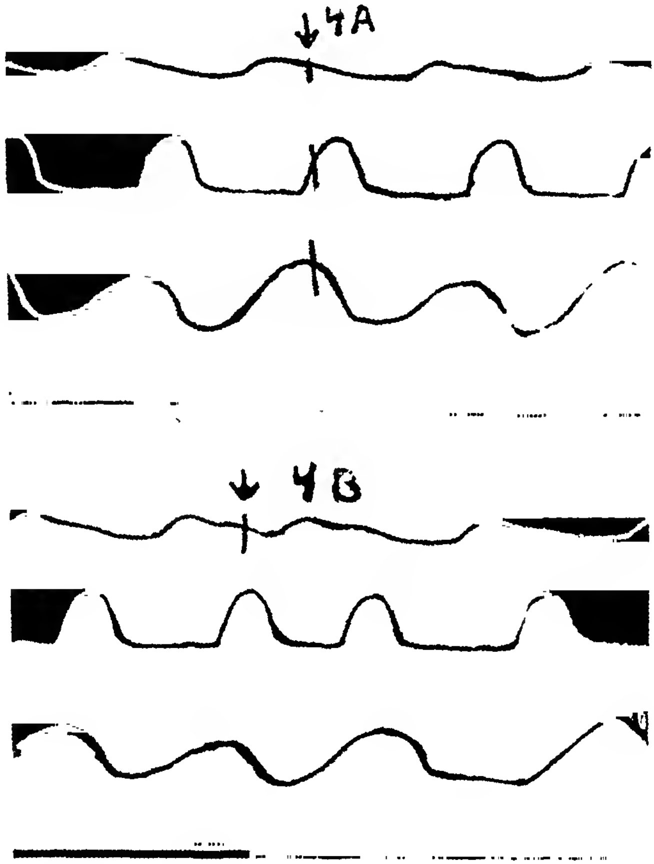


Fig. 4.—Heart rate, 21 per minute. Tracings and coil same as in Fig. 1.

A, A stimulus applied in the middle of the cycle had no effect. The relative refractory period of the sinus in this tortoise was longer than usual.

B, A stimulus of the same strength as in *A* applied slightly later in the cycle induced an extra contraction of the sinus which was carried to the auricles and ventricles. The pause that followed was longer than normal.

The increased height of the "postcompensatory" contraction as applied to the ventricle was well discussed by Cushny,[5] who compares it to a staircase phenomenon. In our work it has occurred after sinus stimulation. Fig. 3*A* exhibits it in the ventricle and to some extent in the auricle, while Fig. 1 *A* and *C* shows it occurring in the sinus even though the pause following the extrasystole was either longer or shorter than normal. Fig. 3*A* is of further interest because the compensatory pause in the auricles and in the ventricles was not preceded by an extrasystole, and therefore the factors leading to the increased height of the "postcompensatory" contraction must have their genesis associated only with the rest period.

DISCUSSION

Among the great number of papers reviewed, those dealing with electrical stimulation of the heart[6-8] have presented a mass of material and illustrations that bear on our results, yet they do not show records of sinal contraction. The original figures of Marey[9] showing the compensatory pause of the ventricle are still the classical illustrations in this field. Schultz[10] has studied records of the isolated sinus and auricles. With these researches as a background, Lewis[3] discussed and showed diagrammatically the condition of the sinus, auricles, and ventricles during the extra discharges and other irregularities of the pacemaker. Our records taken simultaneously from the sinus, auricles, and ventricles confirm and extend the diagrams of Lewis[3] and others.[11-13]

All the records shown (Figs. 1 to 4) were obtained several times so that we feel that the results illustrated by these figures are not incidental to the technique but represent the actual activity of the sinus. Also we have obtained at room temperature reproductions of all the records obtained with cooled preparations, thus demonstrating that the results obtained with cooled hearts were not bizarre due to the cooling of the heart as Cushny[5] long ago warned against.

One of the most frequent questions asked by students is what happens to the rhythmic discharge of the sinus following an extra stimulus and systole of the sinus? We have answered this by careful measurements of time relations and can therefore say that the time in the cycle when the stimulus is applied usually determines the length of the pause preceding the next sinus discharge. The next most important factor is the condition of the tortoise. All of the records illustrated as well as the data in Table I were taken from fresh preparations in which relatively weak stimuli gave satisfactory results. In addition, many records were taken from tortoises in poor condition; that is, several hours after pithing, preparing, and use. These data showed the refractory and relative refractory periods were longer than normal and that an extrasystole placed as early in the cycle as the sinus would permit was almost always followed by a longer pause than normal.

Thus it is obvious that the pause following an extra stimulus to the sinus, although variable, is predictable in length and probably depends somewhat upon the physiologic condition of the cells responsible for the discharge but mostly on the time of stimulation. With these factors in mind the stimulus may be placed so as to give an extrasystole and pause which will show a fairly consistent pattern when repeated records are taken from the same tortoise.

SUMMARY

Graphic records of the contractions of the sinus venosus of turtles were obtained simultaneously with contractions of the auricles and ventricles.

An electrical stimulus applied during the refractory period of the sinus will not cause an extrasystole, but the rhythm may be disturbed.

Electrical stimuli applied to the sinus during its quiescent period caused a contraction which may or may not be carried in turn to the auricles and ventricles.

The pause in the sinus (returning cycle) following an extra or induced contraction of the sinus may be shorter, longer, or equal to normal. The factors determining the length of this pause are governed to some extent by the physiologic condition or property of the cells in the sinus but mostly by the time in the cycle when the extrasystole is induced. With these factors in mind, the sinus of any tortoise will exhibit somewhat predetermined temporal discharges following electrical stimulation. This was indicated by the fact that an extrasystole occurring in early diastole was usually followed by a pause of shorter duration than normal, while one occurring near the end of the cycle was usually followed by a pause of longer duration than normal.

The height of the next contraction of the sinus after an extrasystole of the sinus may be higher than normal even though the pause preceding it was shorter than normal.

The strength of the stimulus may be changed from threshold to considerably above threshold without affecting the results.

REFERENCES

1. Wiggers, C. J.: Physiology in Health and Disease, ed. 2, Philadelphia, 1937, Lea & Febiger.
2. Wiggers, C. J.: Circulation in Health and Disease, ed. 2, Philadelphia, 1923, Lea & Febiger.
3. Lewis, T.: The Mechanism and Graphic Registration of the Heart Beat, ed. 3, Baltimore, 1925, Lea & Febiger.
4. Engelmann, T. W.: Ueber den Ursprung der Herzbewegung und die physiologischen Eigenschaften der grossen Herzvenen des Frosches, Arch f. d. ges. Physiol 65: 109, 1896.
5. Cushny, A. R.: Stimulation of the Isolated Ventricle, With Special Reference to the Development of Spontaneous Rhythm, Heart 3: 257, 1912.
6. McWilliams, J. A.: On the Rhythm of the Mammalian Heart, J. Physiol. 9: 167, 1888.
7. Cushny, A. R., and Matthews, S. A.: On the Effects of Electrical Stimulation of the Mammalian Heart, J. Physiol. 21: 213, 1897.
8. Lewis, T., and White, P. D.: The Effects of Premature Contractions in Vagotimised Dogs, With Especial Reference to Atrioventricular Rhythm, Heart 5: 335, 1914.
9. Marey, M.: Recherches sur les excitations electriques du coeur, J. d'Anat. et Physiol. 13: 60, 1877.
10. Schultz, W. H.: Studies in Heart Muscle. The Refractory Period and the Period of Varying Irritability, Am. J. Physiol. 22: 133, 1908.
11. Rihl, J.: Zur Erklärung der Vergrösserung der postextrasystolischen Systole des Säugetierherzens, Ztschr. f. exper. Path. u. Therap. 3: 1, 1906.
12. Hirschfelder, A. D., and Eyster, J. A. E.: Extrasystoles in the Mammalian Heart, Am. J. Physiol. 18: 222, 1907.
13. de Boer, S.: On the Artificial Extrapause of the Ventricle of the Frog's Heart, Am. J. Physiol. 57: 179, 1921.

CHEMICAL

A CONVENIENT ARRANGEMENT FOR RAPID DIALYSIS*

WALTER H. SEEGERS, PH.D., IOWA CITY, IOWA

THE apparatus in Fig. 1 is designed (1) to give a large amount of membrane surface per cubic centimeter of solution to be dialyzed, and (2) to clear away efficiently the material which diffuses through the membrane. It is inexpensive and there are no difficult technical problems to consider.

The material to be dialyzed (*A*) is placed in a seamless cellulose dialysis tube (*B*) (Visking Corporation, Chicago). Most of the space in this tube is filled with a cylindrical glass plug (*C*), which is made from ordinary glass tubing. If the dimensions of the latter are properly selected, the liquid to be dialyzed will be evenly distributed in thin layers over a large area of membrane

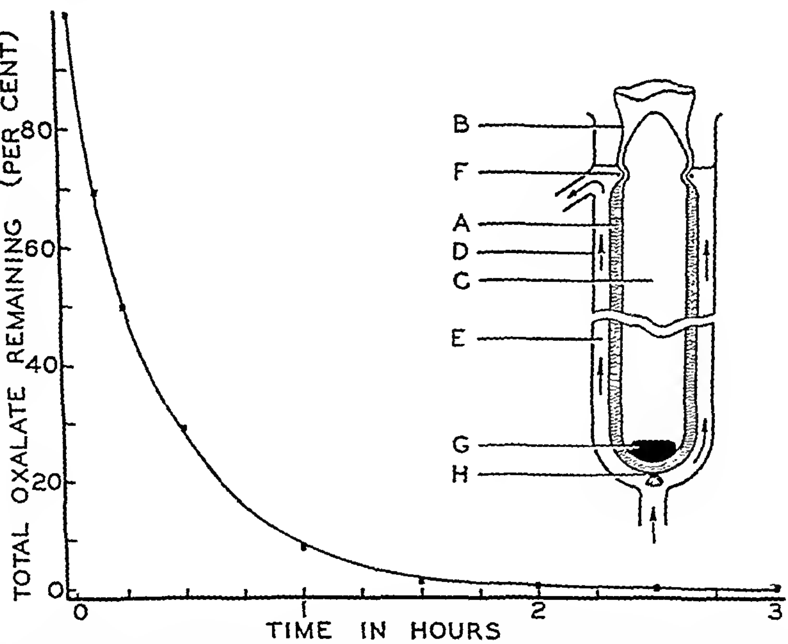


Fig. 1.—Rate of dialysis when a 2 per cent oxalic acid solution is dialyzed against water. *A*, Solution to be dialyzed; *B*, dialysis membrane; *C*, glass plug; *D*, outer glass cylinder; *E*, solution to be dialyzed against; *F*, rubber band; *G*, mercury weight; and *H*, half hitch knot at end of dialysis membrane. Drawing made by H. Kritzler.

surface. The molecules will have only a short distance to travel and the proportionately large surface area supplies an abundant number of pores. The plug is suitably weighted, with mercury for example, and the entire arrangement can be floated in water or any other solution against which one wants to dialyze. To facilitate clearing away material which diffuses through the membrane, the

*From the Department of Pathology, State University of Iowa, Iowa City.
Received for publication, June 22, 1942.

The pause in the sinus (returning cycle) following an extra or induced contraction of the sinus may be shorter, longer, or equal to normal. The factors determining the length of this pause are governed to some extent by the physiologic condition or property of the cells in the sinus but mostly by the time in the cycle when the extrasystole is induced. With these factors in mind, the sinus of any tortoise will exhibit somewhat predetermined temporal discharges following electrical stimulation. This was indicated by the fact that an extrasystole occurring in early diastole was usually followed by a pause of shorter duration than normal, while one occurring near the end of the cycle was usually followed by a pause of longer duration than normal.

The height of the next contraction of the sinus after an extrasystole of the sinus may be higher than normal even though the pause preceding it was shorter than normal.

The strength of the stimulus may be changed from threshold to considerably above threshold without affecting the results.

REFERENCES

1. Wiggers, C. J.: Physiology in Health and Disease, ed. 2, Philadelphia, 1937, Lea & Febiger.
2. Wiggers, C. J.: Circulation in Health and Disease, ed. 2, Philadelphia, 1923, Lea & Febiger.
3. Lewis, T.: The Mechanism and Graphic Registration of the Heart Beat, ed. 3, Baltimore, 1925, Lea & Febiger.
4. Engelmann, T. W.: Ueber den Ursprung der Herzbewegung und die physiologischen Eigenschaften der grossen Herzvenen des Frosches, Arch f. d. ges. Physiol 65: 109, 1896.
5. Cushny, A. R.: Stimulation of the Isolated Ventricle, With Special Reference to the Development of Spontaneous Rhythm, Heart 3: 257, 1912.
6. McWilliams, J. A.: On the Rhythm of the Mammalian Heart, J. Physiol. 9: 167, 1888.
7. Cushny, A. R., and Matthews, S. A.: On the Effects of Electrical Stimulation of the Mammalian Heart, J. Physiol. 21: 213, 1897.
8. Lewis, T., and White, P. D.: The Effects of Premature Contractions in Vagotimised Dogs, With Especial Reference to Atrioventricular Rhythm, Heart 5: 335, 1914.
9. Marey, M.: Recherches sur les excitations electriques du coeur, J. d'Anat. et Physiol. 13: 60, 1877.
10. Schultz, W. H.: Studies in Heart Muscle. The Refractory Period and the Period of Varying Irritability, Am. J. Physiol. 22: 133, 1908.
11. Rihl, J.: Zur Erklärung der Vergrösserung der postextrasystolischen Systole des Säugetierherzens, Ztschr. f. exper. Path. u. Therap. 3: 1, 1906.
12. Hirschfelder, A. D., and Eyster, J. A. E.: Extrasystoles in the Mammalian Heart, Am. J. Physiol. 18: 222, 1907.
13. de Boer, S.: On the Artificial Extrapause of the Ventricle of the Frog's Heart, Am. J. Physiol. 57: 179, 1921.

A CONVENIENT ARRANGEMENT FOR RAPID DIALYSIS*

WALTER H. SEEGERS, PH.D., IOWA CITY, IOWA

THE apparatus in Fig. 1 is designed (1) to give a large amount of membrane surface per cubic centimeter of solution to be dialyzed, and (2) to clear away efficiently the material which diffuses through the membrane. It is inexpensive and there are no difficult technical problems to consider.

The material to be dialyzed (*A*) is placed in a seamless cellulose dialysis tube (*B*) (Visking Corporation, Chicago). Most of the space in this tube is filled with a cylindrical glass plug (*C*), which is made from ordinary glass tubing. If the dimensions of the latter are properly selected, the liquid to be dialyzed will be evenly distributed in thin layers over a large area of membrane

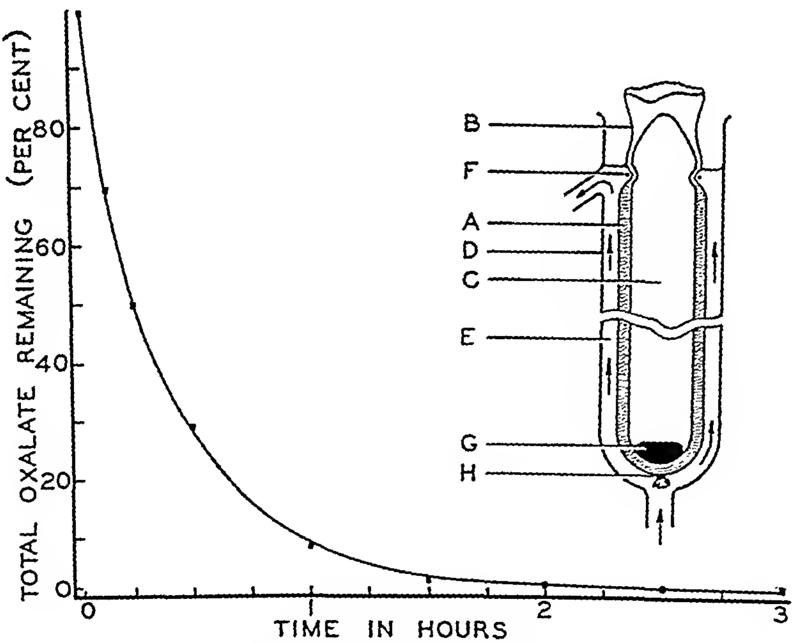


Fig. 1.—Rate of dialysis when a 2 per cent oxalic acid solution is dialyzed against water. *A*, Solution to be dialyzed; *B*, dialysis membrane; *C*, glass plug; *D*, outer glass cylinder; *E*, solution to be dialyzed against; *F*, rubber band; *G*, mercury weight; and *H*, half hitch knot at end of dialysis membrane. Drawing made by H. Kritzler.

surface. The molecules will have only a short distance to travel and the proportionately large surface area supplies an abundant number of pores. The plug is suitably weighted, with mercury for example, and the entire arrangement can be floated in water or any other solution against which one wants to dialyze. To facilitate clearing away material which diffuses through the membrane, the

*From the Department of Pathology, State University of Iowa, Iowa City.
Received for publication, June 22, 1942.

float is confined to a cylinder (*D*) through which a constant flow of liquid (*E*) can be maintained by gravity.

Dimensions of the assembly are arbitrary and within limits can be varied considerably, especially in length. Considerable use has been made of one suitable for dialyzing 25 c.c. of material. The outside dimensions of the cylindrical plug (*C*) are 1.91 × 21.5 cm., and the diameter of the dialysis membrane is listed as 29/32 inch inflated diameter by the manufacturer. In operation the thickness of the material to be dialyzed is about 0.16 cm., and each cubic centimeter is exposed to a surface area of approximately 6.0 sq. cm.

To illustrate (curve on Fig. 1) the characteristics of the apparatus with the above dimensions 25 c.c. of a 2 per cent solution of oxalic acid were dialyzed against water. The water flow through the cylinder D was 100 c.c. per minute. At the end of fifteen minutes half of the oxalate had dialyzed through the membrane, most of it had disappeared within the hour, and only small amounts remained at the end of three hours. Without the glass plug approximately 10 per cent will remain at the end of two hours. If the water flow through the cylinder is reduced to 50 c.c. per minute or increased to 600 c.c. there is no appreciable change in the dialysis rate.

ROUTINE ANALYSIS OF URINARY CALCULI*

A Rapid, Simple Method Using Spot Tests

Julius H. Winer,† M.D., and Marjorie R. Mattice, M.S.
New York, N. Y.

URINARY stone analysis is an important factor in determining the etiology of a stone and subsequent management of the patient. The common "systems" of stone analysis have often been regarded with misgivings since they appear to be too complicated, time-consuming, or otherwise unsatisfactory from the standpoint of the urologist. We have, therefore, compiled simple chemical tests whereby a dependable analysis may be made in the ordinary office laboratory in a few minutes by a worker without special training. A further advantage is that several calculi may be examined simultaneously without additional effort.

Reagents.—The appended list of reagents includes several which are not essential, since alternative methods are given to allow for individual choice. If these solutions are kept in dropper-type bottles, their use is facilitated, since only minute amounts are needed for testing.

Uric acid reagent: Consult textbooks for the preparation of the Benedict or Folin-Denis reagents used for urine and blood uric acid determinations.

*From the Department of Urology and the Division of Pathological Chemistry, Department of Medicine, New York Post-Graduate Medical School of Columbia University.
Received for publication, July 10, 1942.
†Now Lieutenant Medical Corps, U. S. Army.

Sodium carbonate: 20 per cent solution.

Ammonium molybdate: Several different solutions may be employed but that suggested by Seifter and Trattner[10] is excellent. Dissolve 3.5 Gm. of ammonium molybdate in 75 c.c. of water and pour into 25 c.c. of concentrated nitric acid.

Manganese dioxide: A heavy, fine, black powder, MnO_2.

Resorcinol or resorcin: Pure white needlelike crystals $C_6H_4(OH)_2$.

Hydrochloric acid: 10 per cent solution (concentrated acid diluted ten times).

Sulfuric acid: Concentrated H_2SO_4.

Sodium hydroxide: 20 or 25 per cent solution.

Ammonium hydroxide: 28 per cent (concentrated).

Reagent "M": Dissolve 1 mg. of p-nitrobenzene-azoresorcinol in 100 c.c. of normal sodium hydroxide (Eastman).

Nessler's solution: Consult textbooks for the preparation of the Bock-Benedict or Folin formula for blood analyses.

Sodium cyanide: Prepare a 5 per cent solution and preserve with 1 c.c. of 28 per cent ammonium hydroxide per 500 c.c. of solution.

Sodium nitrite: A fresh solution, 0.1 per cent, should be prepared once a week, although it often keeps much longer.

Sodium nitroprusside (nitroferricyanide): Prepare a 5 per cent solution and discard when it shows signs of fading. Protect from light.

Reagent "S": Dissolve 100 mg. of N-(1-naphthyl)ethylenediamine dihydrochloride (La Motte Chemical Products Company) in 100 c.c. of water. Store in an amber bottle.

Reagent "S" (alternative): Dilute 1 c.c. of dimethyl-α-naphthylamine (Eastman) in 250 c.c. of 95 per cent alcohol.

Nitric acid: Concentrated (unnecessary unless murexide supplementary test is employed).

Procedures.—The general scheme of analysis is presented in tabulated form. The calculus should be split so as to reveal different structures within, and the core or nucleus should be distinguished from the various layers by separate analysis. For full evaluation of the results the relative sensitivity of the various tests should be taken into account. The findings depend largely on the methods used to detect possible constituents of the stone. Since the calculus is constantly bathed in urine during its formation, traces of every substance found therein would be discovered in the stone if sufficiently delicate procedures were employed. The purpose of our scheme of analysis is to reveal the chief components of the stone, the minor constituents being of research rather than practical importance insofar as the present state of knowledge is concerned.

When the reagents for the Benedict-Theis method of determining serum *phosphate* are used on urinary calculi, they give a "positive" reaction with a majority of stones, including some thought to be "pure" cystine. It is, therefore, impractical to employ such a test.

The murexide test for *uric acid* is less sensitive than the recommended reagents, although it must be stressed that only a deep blue color should be re-

TABLE I

SCHEME OF ANALYSIS OF URINARY CALCULI

Chemical Group	*Reagents Added*	*Results*
1. Urates	Pulverized stone 1 drop Na_2CO_3 2 drops uric acid reagent	Prompt deep *blue* color (pale blue is negative)
2. Phosphates*	Pulverized stone 4-5 drops ammonium molybdate solution (need excess of reagent)	Warm over flame to get distinct *yellow precipitate,* $(NH_4)_3PO_4 \cdot 12\ MoO_3$
3. Oxalates	Pulverized stone Pinch of resorcinol 1 drop conc. H_2SO_4 Mix	Slow development of dull *bluish-green* color (Higgins and Mendenhall[5])
4. Oxalates	Pulverized stone 2-3 drops HCl. If no effervescence, add pinch of MnO_2. Do not mix	Tiny *bubbles of gas* "explosively" released from bottom
5. Carbonates	Relatively large sample of pulverized stone 8-10 drops HCl	*Effervescence*
Take up acid extract from *5* in aspirating pipette the tip of which is lightly plugged with cotton. Remove cotton by seizing projecting wisp and divide "filtrate" into three aliquots for *6, 7,* and *8.*		
6. Calcium*	Acid extract 2-3 drops NaOH	Fine *white* precipitate or film from oxalate stones; dense precipitate from phosphate stones
7. Magnesium	Acid extract 2-3 drops NaOH 2-3 drops reagent "M"	Reddish-purple reagent slowly becomes definitely *blue* (precipitate forms)
8. NH_4 group	Acid extract 2-3 drops NaOH 2-3 drops Nessler's solution *Alternative:* Pulverized stone 2-3 drops Nessler's solution	*Yellowish-orange* precipitate
9. Sulfonamides	Pulverized stone 2 drops HCl (wait 30 seconds) 2 drops $NaNO_2$ (wait 30-60 seconds) 2-3 drops reagent "S"	*Brownish-pink* to *magenta*
10. Sulfonamides	Pulverized stone 8-10 drops HCl Stir with broken piece of match stick or wooden applicator Let stick remain *in situ* for several minutes	Lignin in wood becomes *yellow* or *orange.* Use stick for "writing" on paper towel or newspaper. The colorless acid extract turns *yellow* on lignin-containing paper but not on rag stock (Halley[3])
11. Cystine	Pulverized stone 1 drop NH_4OH 1 drop NaCN (wait 5 minutes) 2-3 drops sodium nitroprusside	*Beet-red* color; on standing may fade to orange red

*Use a microscope slide for these tests, spot plate for the others. The artist's type is more satisfactory than the regular chemist's spot plate.

garded as "positive" (test 1). The murexide test, however, is much more useful and informative as shown later.

Nessler's solution (test 8) will detect smaller amounts of *ammonia* than will pink litmus paper when exposed to the gas released by treatment of an acidified extract of the calculus with sodium hydroxide.

The Marshall reagents will find smaller amounts of *sulfonamides* than will the lignin test of Halley. A relatively large sample of stone should be used for the latter procedure; even then the presence of an appreciable quantity of sulfonamide may not be detected.

Suitable tests for *oxalate* are not as sensitive or as specific as one might wish. In the experience of one of us (M. R. M.) the conversion of oxalate to carbonate by manganese dioxide has proved highly satisfactory (test 4). When only a minute fragment is available, oxalates can be found by using a narrow test tube and 5 to 10 drops of acid, and watching carefully with a magnifying lens for the ejection of a few bubbles of gas on addition of the black powder. This test for oxalates is not applicable to carbonate stones until all carbon dioxide has been removed from an acid extract by boiling. The solution must be cool before the manganese dioxide is added. However, we have rarely encountered carbonate in interfering amounts in oxalate stones, although Kamlet[5] reports finding carbonate in 12 out of 88 oxalate stones. With moderate-sized samples the test is easily carried out on a white or clear-glass spot plate if the black powder is not mixed after it falls upon the particles of stone. After a moment's hesitation tiny bubbles of gas are shot out like rockets. The effervescence seen after contact of hydrochloric acid with carbonate is different; the powdered stone is buoyed up by the immediate formation of many very fine bubbles of carbon dioxide. The Higgins-Mendenhall[4] test for oxalates (test 3), while specific and simple to perform, produces a poor color and that rather slowly, and requires a fairly generous test sample for easy recognition of oxalates.

Physical Characteristics.—Instances occur where the stone is minute or only a small part of it is available for study, necessitating the use of a few selected tests dependent on the examiner's ingenuity. Fortunately, the appearance of a stone generally gives a clue to its composition.

Uric acid often occurs as multiple, smooth, round pebbles, which are devoid of luster and vary in size from a few millimeters to 30 mm. or more in diameter. Single stones frequently have a bumpy or eruptive type of surface, resembling miniature volcanic craters. Uric acid stones are always definitely yellow, but this color may not be noted until the stone is crushed.

Phosphate and *carbonate* stones occur as compact balls (often several centimeters in diameter) or as large friable masses assuming the shape of the cavity were found; these are clay or chalk-like. Rarely, triple phosphate stones ($MgNH_4PO_4$) assume porous, coral-like formations, which suggest calcium oxalate except for their whiteness.

Oxalate stones show considerable variety in form. Irregular, light-colored fragments may exhibit elaborate crystalline structures with sharp projecting blades. Oxalate stones are conspicuous for their crystalline glint or smooth luster. The dark-brown type is very compact and may take a grape-seed or berry-like form, which should be differentiated from the prostatic corpora amylacea. If the rounded contours are larger, like a bunch of grapes, the calculi may be described as botryoidal. Although the berry and compact stratified forms are dark brown, crushing produces a light-colored powder. Mammillated

oxalate stones occur, although the nipplelike structure is rarer than a prickly surface, resembling the "eruptive" type of uric acid calculus. Buff-colored or brown stones may be covered with a fine coral-like excrescence. Occasionally, the whole structure is loose and porous.

Cystine stones occur largely as pale yellow or white granules upon which the pestle seems to skid as it crushes the calculus. Multiple stones may develop in the form of uric acid pebbles, except that they are usually much smaller, many hardly reaching 1 mm. in diameter. Such stones possess a faint waxy luster and are brownish in color which distinguishes them from the yellow uric acid pebbles. Another form of cystine stone resembles calcium oxalate on casual inspection since it presents a crystalline surface.

The *sulfonamides* may be deposited on the surface of uric acid without obvious layering or may be mingled with phosphate and carbonate in a putty-like mass. There is nothing in the physical appearance of the stone to suggest the presence of sulfonamides. It is yet to be determined whether or not "pure" sulfonamide calculi will occur.

It is important to analyze prominent layers of calculi when possible. Although uric acid stones often are "pure," they frequently pick up a thick crust or coating of different nature, usually phosphates unless sulfonamides are available. Phosphates do not, as a rule, deposit as a visible crust on oxalate stones but occur throughout the calculus. Instances are seen where uric acid encircles an oxalate nucleus several millimeters in diameter and then is thickly encased in magnesium ammonium phosphate and calcium carbonate.

Supplementary Tests.—Tests other than those described in the table may be desired by the technician for guidance or for checking purposes. The following tests are valuable in that they furnish a lead as to the presence of substances other than those sought.

Murexide Test.—When a sample of pulverized stone is treated with several drops of concentrated nitric acid and carefully evaporated to dryness, then subjected to 2-3 drops of ammonia water, the following may be observed:

Uric acid— deep yellow to orange-red or crimson becoming more purplish with ammonium hydroxide (bluish violet with sodium hydroxide).
Xanthine— greenish-yellow turning to orange with ammonium hydroxide becoming reddish on warming (deep orange-red with sodium hydroxide).
Protein— pale yellow turning to orange.
Sulfonamides—yellow turning to mahogany brown.

It should be emphasized that the murexide test for uric acid usually is carried out in an evaporating dish on a water bath, but this may not provide a sufficiently high temperature for the xanthine reaction. It is very easy to miss xanthine in urinary calculi.

According to commonly used "systems" of stone analysis, no gas is evolved when concentrated nitric acid is added to a pulverized uric acid calculus, whereas effervescence occurs in the presence of ammonium urate. Domanski[1] emphasizes this point and further states that uric acid is not appreciably soluble in nitric acid, although urates dissolve readily. In our experience both uric acid and urates are soluble under these conditions. Uric acid, whether reagent pure, re-

covered from a lateritious deposit or obtained in calcareous form, reacts with concentrated nitric acid so as to release a continuous stream of bubbles until the powder is no longer visible. In the case of stones, the effervescence is quite frothy whereas it is quieter and confined to a swirling motion within the fluid when pure uric acid is tested, but this may be due solely to the fact that the reagent grade acid exists in a more finely divided form than does the crudely ground calculus. All stones giving a positive murexide test have shown this gas formation whether ammonium urate was present or not.

In this connection a further point needs clarification. The standard method for testing for the ammonium radical in urinary stones involves the addition of strong alkali for the release of ammonia gas and its detection by wet pink litmus paper or filter paper treated with Nessler's solution. Domanski found that ammonia was not obtained from ammonium urate by this process. We have had no difficulty in distinguishing between ammonium urate and uric acid stones by the method described in this paper (test 8). However, ammonium urate stones appear to be relatively rare—which may be less fact than failure to discover the ammonium radical.

Ashing Process.—Another helpful procedure is to ash a sample of the stone in a porcelain crucible. This is a desirable preliminary to the test for phosphates given in the table (test 2) but is not essential. It is difficult to evaluate the odor of the heated stone except in the case of cystine. This penetrating odor, once experienced, is never forgotten. It follows the first curl of smoke and diminishes in intensity with increased heat. Both cystine and urate stones blacken rapidly, the former burning "clean" and the latter with advancing greasy brown rings up the crucible; both leave the crucible empty. The residue from phosphate and oxalate stones is of about the same bulk as the original sample. The phosphates rarely give a white ash even after long and hard heating. With oxalates the ash is more nearly white and effervesces if acid is added as in testing for phosphorus since oxalate is converted into carbonate by heating. This effervescence is not to be confused with spattering obtained on adding a fluid to the hot ash. Sulfonamides melt and form a black tarry mass which is resistant to ashing. It can be seen, then, that the response to heating may furnish valuable clues as to the composition of a calculus.

SUMMARY

1. A scheme of urolith analysis is presented which is simple, rapid, and dependable.

2. A number of calculi or separate layers can be analyzed simultaneously with the simple equipment required.

3. The physical characteristics of urinary stones and supplementary means for testing their composition are discussed.

Appreciation is expressed to Dr. Joseph A. Hyams for his stimulating interest in this investigation.

REFERENCES

1. Domanski, T. J.: Renal Calculi: A New Method for Qualitative Analysis, J. Urol. 37: 399, 1937.
2. Feigl, F.: Qualitative Analysis by Spot Tests. Translated by J. W. Matthews, ed. 2, New York, 1939, Nordemann Publishing Co.

3. Halley, L. I.: Lignin Tests for Sulfonamides, Virginia M. Monthly 69: 334, 1942.
4. Higgins, C. C., and Mendenhall, E. E.: A Qualitative Test for Sulfapyridine and Its Derivatives in Urinary Calculi, J. Urol. 42: 450, 1939.
5.—: Factors Associated With Recurrent Formation of Renal Lithiasis With Report of New Methods for Qualitative Analysis of Urinary Calculi, Ibid. 42: 436, 1939.
6. Kamlet, J.: Analysis of Renal Calculi, J. Lab. & Clin. Med. 23: 321, 1937.
7. Marshall, E. K., Jr.: Determination of Sulfanilamide in Blood and Urine, J. Biol. Chem. 122: 263, 1937.
8. —, and Bratton, A. C.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. 128: 537, 1939.
9. Mattice, M. R.: Chemical Procedures for Clinical Laboratories, Philadelphia, 1936, Lea & Febiger.
10. Seifter, J., and Trattner, H. R.: Simplified Qualitative Analysis of Urinary Calculi by Spot Tests, J. Urol. 42: 452, 1939.

A CRITICAL STUDY OF THE CARR-PRICE REACTION FOR THE DETERMINATION OF β-CAROTENE AND VITAMIN A IN BIOLOGICAL MATERIALS*

Margaret Kaser, Ph.D., and Jakob A. Stekol, D.Sc., Nashville, Tenn.

SEVERAL methods based on the Carr-Price[1] reaction for the determination of β-carotene and vitamin A in biological materials have been described and widely applied to nutritional studies.[3, 7-9, 11, 13] The limitations of the reaction have been pointed out on several occasions[3, 7] and summarized recently[12] with the conclusion that under properly controlled conditions the antimony trichloride reaction with β-carotene and vitamin A is suitable for some studies, although spectrophotometric and biological assays give more reliable results. Sakamoto[14] found good agreement between values for β-carotene and vitamin A obtained by polarimetric and colorimetric methods. Embree[4] finds that the measurement of the color produced by antimony trichloride in the presence of vitamin A by the use of the photoelectric colorimeter is almost as exact as that given by the spectrophotometer. Salter,[15] on the other hand, in a recent review stated that the methods for vitamin A based on the Carr-Price reaction are too erratic for routine use.

We have had occasion in this laboratory to use the Carr-Price reaction for the analysis of a large number of samples of blood plasma or serum, vitamin concentrates, and rat tissues. In view of the recent criticism of the reaction, it appeared important to establish the validity of the results obtained by such a procedure. Therefore, the present work was undertaken.

EXPERIMENTAL

Two modifications of the Carr-Price reaction, both of which involve the use of the Evelyn colorimeter, have been studied. One is the micromethod described in detail by May, Blackfan, McCreary, and Allen,[10] and the other is

*From the Department of Biochemistry, Vanderbilt University Medical School, Nashville.
Received for publication, July 13, 1942.

the macroprocedure outlined briefly by Lewis, Bodansky, and Haig.[9] The two methods were compared for the reproducibility of results on different and on the same samples of blood plasma. Since the macroprocedure was found satisfactory in our hands, we shall describe it in detail. Recovery studies of crystalline β-carotene and vitamin A employing the macroprocedure were also made. Although Embree[4] found that serious loss of vitamin A potency results from exposure of solutions of vitamin A in clear glass test tubes to ultraviolet light for four to five and a half hours, we used throughout only clear glass glassware. Since the average time necessary to complete the determinations was an hour or less, the excellent recoveries obtained indicate that the destructive influence of ultraviolet light under the conditions used was not in evidence. However, when a large number of samples are run at the same time, requiring several hours for the completion of the determinations, Embree's[4] suggestion to use amber glassware to prevent the destruction of vitamin A by ultraviolet light becomes of practical importance, particularly in the case of samples of low vitamin A potency.

MACROPROCEDURE. BLOOD PLASMA OR SERUM

Five milliliters of 95 per cent alcohol and 8 ml. of petroleum ether (B.P. 35° to 75° C.) are added to 3 to 5 ml. samples of blood plasma or serum, and the tubes are shaken on a mechanical shaker for ten minutes. After centrifuging two or three minutes at 1,300 r.p.m., 6 ml. of the petroleum ether layer are transferred to a previously calibrated colorimeter tube, and the carotene reading is taken with filter 440. The Evelyn tube is immersed in a water bath at about 65° C. and is connected to a water pump. Evaporation is complete in four or five minutes. Prolonging the time of evaporation to ten or fifteen minutes was found to lower the vitamin A values by about 10 per cent. The residue is dissolved in 0.6 ml. of chloroform, and with the tube inserted in the colorimeter, 5.4 ml. of 25 per cent antimony chloride in chloroform are added as quickly as possible. The minimum galvanometer reading with filter 620 is taken within three seconds before the color developed begins to fade.

The carotene values are calculated with the constants of Koehn and Sherman,[7] and for vitamin A we have used the formula of Dann and Evelyn[3] and the factor of 2,100 given by Holmes and Corbet[5] for converting $E\,\frac{1\%}{1\text{ cm.}}$ (328 mμ) to international units.

$$\frac{L_{440} \times 2.65 \times 6 \times 100}{\frac{3}{4} \times \text{ml. of sample}} = \text{Micrograms of carotene per 100 ml.}$$

$$\left[\frac{L_{620}}{\frac{3}{4} \times \text{ml. of sample}} - \frac{0.14\ L_{440}}{\frac{3}{4} \times \text{ml. of sample}}\right] \times 52.5 \times \frac{6}{100} \times 0.41 \times 2{,}100 = \text{I. U. vitamin A per 100 ml.}$$

RAT LIVER

One gram samples of liver are heated with 5 ml. of saturated potassium hydroxide in 95 per cent alcohol at 75°-80° C. for forty-five minutes with occasional shaking. After cooling, the digests are made up to 10 ml. with water.

For liver samples containing about 100 to 1,000 international units per gram; an aliquot of 0.2 ml. is taken for analysis. The sample is diluted to about 5 ml. with water and from this point the determination is carried out in the same way as was described for blood plasma.

Applying this procedure to the livers of rats maintained on diets of various nutritional qualities, we obtained concordant results with satisfactory reproducibility.[14]

DISCUSSION

Table I shows data obtained by the microprocedure of May, Blackfan, McCreary, and Allen[10] and the procedure described above. The objective was to check the reproducibility of the values obtained by both procedures on different samples of blood plasma. Table II illustrates the analytical values obtained by both procedures on the same samples. It will be seen from the data presented that the macroprocedure described above gives consistently better and more reproducible values than the microprocedure. We believe that this difference in the performance of the procedures is not due to any dif-

TABLE I

REPRODUCIBILITY OF RESULTS FOR β-CAROTENE AND VITAMIN A IN HUMAN BLOOD PLASMA BY THE MICROPROCEDURE AND MACROPROCEDURE

	SAMPLE I		SAMPLE II	
	MICROMETHOD		MACROMETHOD	
	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.
	128.9	----	116.1	148.0
	105.8	100.3	121.8	152.1
	141.0	62.6	120.7	153.9
	119.9	79.7	121.8	152.1
	122.9	62.9	118.4	151.0
	116.9	77.1	125.1	168.4
	134.9	64.1	118.4	154.4
	119.9	67.6		
Average	123.8	73.5	120.3	154.3
Maximum deviation from mean	14.5%	36.5%	4.0%	9.1%
Average deviation from mean	6.8%	14.3%	1.9%	2.6%

TABLE II

COMPARISON OF VALUES FOR β-CAROTENE AND VITAMIN A OBTAINED ON THE SAME SAMPLE OF BLOOD PLASMA BY THE MICROPROCEDURE AND MACROPROCEDURE

SAMPLE	MACROMETHOD		MICROMETHOD	
	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.	CAROTENE μG/100 ML.	VITAMIN A I.U./100 ML.
1	168.6	258.2	144.0	227.5
	173.9	318.9	119.9	222.7
	171.3	301.2	138.0	208.0
Average	171.3	292.8	134.0	219.4
2	171.3	224.2	138.0	147.8
	172.6	241.6	----	----
Average	172.0	232.9	138.0	147.8
3	63.7	40.0	43.3	24.1
	63.7	42.9	46.3	----
Average	63.7	41.5	44.8	24.1

ference in the basic principle employed, but to the difficulty in carrying out all the steps involved in a sufficiently short time by the microprocedure as is essential to the accuracy of the reaction.

Table III indicates that no significant increase in the vitamin A resulted after a preliminary saponification of 3 ml. samples of plasma in alcoholic potassium hydroxide for twenty minutes. It appears that most of the vitamin in normal human plasma exists in the free form, as has been suggested previously.[2]

TABLE III

EFFECT OF SAPONIFICATION ON THE β-CAROTENE AND VITAMIN A VALUES OF NORMAL HUMAN BLOOD PLASMA

SAMPLE	CAROTENE μG/100 ML.		VITAMIN A I.U./100 ML.	
	UNSAPONIFIED	SAPONIFIED	UNSAPONIFIED	SAPONIFIED
1	243.2	241.9	130.6	136.5
2	215.8	206.8	131.5	141.1
3	98.8	88.2	153.8	154.6
4	160.8	151.8	128.2	134.0
5	146.8	145.6	107.1	100.4
6	110.6	106.7	127.6	137.6
7	307.7	285.0	122.8	132.0
8	103.1	103.1	119.1	120.7
Average	173.4	166.1	127.6	132.1

TABLE IV

RECOVERY OF CRYSTALLINE β-CAROTENE AND VITAMIN A FROM PETROLEUM ETHER

CAROTENE ADDED μG	CAROTENE FOUND μG	VITAMIN A ADDED μG	VITAMIN A ADDED I. U.*	VITAMIN A FOUND I. U.
0.464	0.484	0.463	1.389	1.470
0.696	0.748	0.695	2.085	1.944
0.928	1.003	0.926	2.778	2.628
1.160	1.265	1.158	3.474	2.700
2.320	2.389	2.316	6.948	6.840
3.480	3.498	3.474	10.422	10.980
4.640	4.650	4.632	13.896	13.560
5.800	5.995	5.790	17.370	18.225
6.960	7.059	6.948	20.844	19.710
8.120	8.203			
9.280	9.240			
10.440	10.380			
11.600	11.290			
12.760	12.240			
13.920	13.450			

*The weights of crystalline vitamin taken were converted to international units by means of the relationship of 3 I. U. per microgram given by Holmes and Corbet.[5]

Recoveries of carotene and vitamin A were made on separate solutions of known concentration. The average deviation from the theoretical values, as calculated from the figures above, was +1.6 per cent for carotene and −1.9 per cent for vitamin A.

Tables IV and V show data illustrating the recovery of crystalline β-carotene and vitamin A from petroleum ether, either separately or when mixed in various proportions. As will be seen, the procedure outlined above gives excellent recoveries with reasonable experimental errors. In Table VI are presented data for the recovery of β-carotene and vitamin A from normal human plasma. Again the recoveries were sufficiently good to warrant the use of the procedure for routine use.

TABLE V

RECOVERY OF MIXTURES OF CRYSTALLINE β-CAROTENE AND VITAMIN A FROM PETROLEUM ETHER

SERIES	CAROTENE ADDED µG	CAROTENE FOUND µG	VITAMIN A ADDED µG	VITAMIN A ADDED I. U.*	VITAMIN A FOUND I. U.
I	0.708	0.728	0.421	1.263	1.434
	2.123	2.103	0.421	1.263	1.341
	3.540	3.557	0.421	1.263	1.170
	4.957	4.692	0.421	1.263	1.218
	6.490	6.201	0.421	1.263	1.110
II	0.708	0.863	3.507	10.521	10.680
	2.123	2.103	3.507	10.521	10.377
	3.540	3.499	3.507	10.521	10.560
	4.957	4.723	3.507	10.521	10.347
	6.490	6.237	3.507	10.521	10.635

*The weights of crystalline vitamin taken were converted to international units by means of the relationship of 3 I. U. per microgram given by Holmes and Corbet.[5]

The average deviation from the theoretical values in series I was −0.2 per cent for carotene and +2.2 per cent for vitamin A; in series II, for carotene it was +10.8 per cent and for vitamin A −0.6 per cent.

TABLE VI

RECOVERY OF CRYSTALLINE β-CAROTENE AND VITAMIN A ADDED TO BLOOD PLASMA

PLASMA LEVELS		CAROTENE		VITAMIN A*		TOTAL RECOVERED	
CAROTENE µG/100 ML.	VITAMIN A I. U./100 ML.	ADDED µG/100 ML.	TOTAL µG/100 ML.	ADDED I. U./100 ML.	TOTAL I.U./100 ML.	CAROTENE µG/100 ML.	VITAMIN A* I. U./100 ML.
120.7	167.5	216.3	337.0	56.1	223.6	335.9	223.6
120.7	167.5	23.6	144.3	280.5	448.0	144.4	451.1
120.7	167.5	118.0	238.7	168.3	335.8	233.2	333.9

*The weights of crystalline vitamin taken were converted to international units by means of the relationship of 3 I. U. per microgram given by Holmes and Corbet.[5]

Petroleum ether solutions of β-carotene and vitamin A of known concentration were added to normal human blood plasma in which the concentrations of carotene and vitamin A were previously determined.

SUMMARY

The applicability of the Carr-Price reaction to the analysis of biological materials has been critically re-examined using the Evelyn photoelectric colorimeter. Excellent recoveries of crystalline β-carotene and vitamin A were obtained with reproducible results. The macroprocedure described was found satisfactory for routine analysis of blood plasma or serum. Preliminary saponification of plasma or serum was found to have no significant effect on the vitamin A or carotene concentrations in normal human plasma. Recent criticisms directed against the Carr-Price reaction as the basis for the quantitative determination of vitamin A and β-carotene in biological materials should take into account the carefully specified conditions which are essential to the successful application of this reaction.

The studies referred to herein were conducted under the auspices of the International Health Division of the Rockefeller Foundation of New York City by the nutrition units of the Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tenn.

REFERENCES

1. Carr, F. H., and Price, E. A.: Colour Reactions Attributed to Vitamin A, Biochem. J. 20: 497, 1926.
2. Clausen, S. W., and McCoord, A. B.: The Distribution of Vitamin A, Carotene and Xanthophyll in the Tissues of Man and Animals, 29th Meeting Fed. Am. Soc. Exper. Biol., Boston, 1942.
3. Dann, W. J., and Evelyn, K. A.: The Determination of Vitamin A With the Photoelectric Colorimeter, Biochem. J. 32: 1008, 1938.
4. Embree, N. D.: Physicochemical Assay of Vitamin A, Indust. & Engin. Chem. Anal. Ed. 13: 144, 1941.
5. Holmes, H. N., and Corbet, R. E.: Isolation of Crystalline Vitamin A, J. Am. Chem. Soc. 59: 2042, 1937.
6. Kaser, M., and Stekol, J. A.: Unpublished data.
7. Koehn, C. J., and Sherman, W. C.: The Determination of Vitamin A and Carotene With the Photoelectric Colorimeter, J. Biol. Chem. 132: 527, 1940.
8. Lewis, J. M., Bodansky, O., Falk, K. G., and McGuire, G.: Vitamin A Requirements in the Rat. The Relation of Vitamin A Intake to Growth and to the Concentration of Vitamin A in the Blood Plasma, Liver and Retina, J. Nutrition 23: 351, 1942.
9. Lewis, J. M., Bodansky, O., and Haig, C.: Level of Vitamin A in the Blood as an Index of Vitamin A Deficiency in Infants and Children, Am. J. Dis. Child. 62: 1129, 1941.
10. May, C. D., Blackfan, K. D., McCreary, J. F., and Allen, F. H., Jr.: Clinical Studies of Vitamin A in Infants and in Children, Am. J. Dis. Child. 59: 1167, 1940.
11. McCoord, A. B., and Luce-Clausen, E. M.: The Storage of Vitamin A in the Liver of the Rat, J. Nutrition 7: 557, 1934.
12. Munsell, H. E.: Vitamin A. Methods of Assay and Sources in Food, J. A. M. A. 111: 245, 1938.
13. Popper, H., and Brenner, S.: Fate of Excess Vitamin A Stores During Depletion. Value of the Histologic Demonstration of Vitamin A, J. Nutrition 23: 431, 1942.
14. Sakamoto, T.: Determination of Vitamin A. I. Colorimetric Method Using the Carr-Price Reaction. II. Polarimetric Method, Kitasato Arch. Exper. Med., Abstracts 17: 3, 1940.
15. Salter, W. T.: The Chemical Measurement and Control of Clinical Vitamin Deficiency, New England J. Med. 226: 649, 688, 1942.

PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM

IV. Relation Between Prothrombin Clotting Time and Plasma Fibrinogen Concentration

Robert C. Page, M.D., Edwin J. de Beer, Ph.D., and Z. Bercovitz, M.D.
New York, N. Y.

THE clinical interpretation of laboratory tests must be based on a knowledge of factors that may cause a variation in the test. The increasing use of the prothrombin clotting time test in studying bleeding and hemorrhage brings up the question of the relation of prothrombin clotting time to the blood concentration of other factors involved in the coagulation mechanism (calcium, thromboplastin, fibrinogen, etc.).

The study reported here consisted of obtaining blood from healthy adults and from patients with chronic ulcerative colitis or miscellaneous gastrointestinal diseases and determining the prothrombin clotting time and the fibrinogen content of the plasma.

METHOD OF STUDY

Blood of healthy normal adults and of patients attending an outpatient clinic was obtained. Most of the blood was obtained in the afternoon so these were mainly postprandial specimens. Blood for determination of the prothrombin clotting time and fibrinogen content was drawn at the same time.

The method employed was Quick's procedure[1] modified by using Russell viper venom instead of brain thromboplastin.[2-4] Four and one-half cubic centimeters of venous blood were drawn into a dry syringe and mixed with 10 mg. of potassium oxalate in a centrifuge tube. The blood was centrifuged at 1,500 r.p.m. for five minutes, and the oxalated plasma was drawn off. The test was performed within two hours after withdrawal of the blood; if hemolysis was present, the sample was discarded. The test was performed as follows: 0.2 c.c. of oxalated plasma was pipetted into a small test tube (75 by 10 mm.), and 0.2 c.c. of Russell viper venom,* 1:10,000 solution, was added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., was then added, and the stop watch immediately started. The tube was agitated for from ten to fifteen seconds and tilted until separate discrete fibrin particles could be seen. The prothrombin clotting time in normal individuals using Russell viper venom has been found to be 20.76 seconds $\pm$ 2.32 seconds[3] and 19.5 seconds $\pm$ 2.9 seconds.[5]

The method used for the fibrinogen content of blood plasma was a combination of the methods of Howe[6] and Wu[7] and Koch and McMeekin[8] as

From the Gastroenterology Clinic, Department of Medicine, New York Postgraduate Medical School and Hospital.

Received for publication, July 16, 1942.

*Supplied as "Stypven" Russell Viper Venom by The Burroughs Wellcome & Co. (U. S. A.), Inc., New York, N. Y.

adopted in the Biochemical Laboratories of the Philadelphia General Hospital. One cubic centimeter of oxalated plasma was diluted with 48 c.c. of physiologic saline. One cubic centimeter of calcium chloride was then added and the mixture was allowed to stand for thirty minutes. The clot was removed, dried, and placed in an ignition tube with 1 c.c. of acid digestion mixture. Five drops of caprylic alcohol were added to prevent foaming. The mixture was boiled vigorously and when white fumes appeared, superoxol (30 per cent H_2O_2) was added to clear the solution (about 0.5 c.c.). The solution was again heated vigorously and then cooled. Distilled water was added to make a total volume of 35 c.c. Fifteen cubic centimeters of Nessler's solution were then added, the solution was centrifuged, and the supernatant fluid was matched against standards containing from 0.6 to 1.0 mg. of nitrogen. The normal range for plasma fibrinogen with this procedure is 0.2 to 0.4 Gm. per 100 c.c.

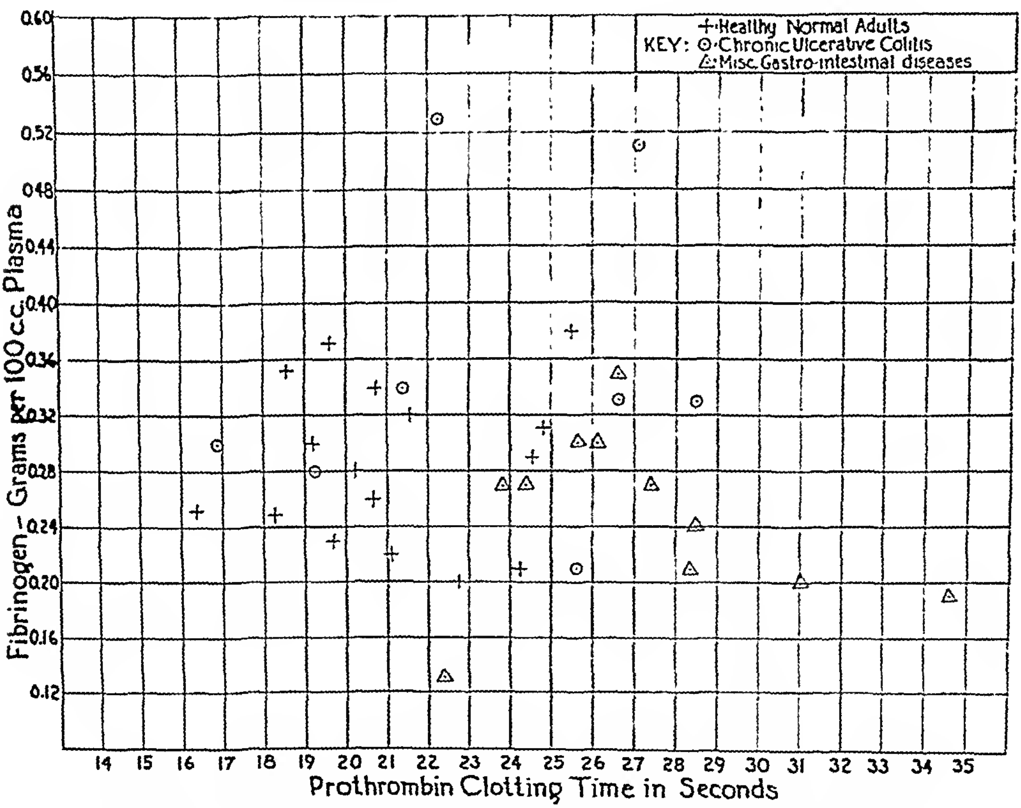


Fig. 1.—Chart showing relation between prothrombin clotting time and plasma fibrinogen in normal adults and in patients with gastrointestinal disorders.

DISCUSSION OF RESULTS

The results are plotted in Fig. 1. It will be observed that the points are widely scattered, and there is little evidence of any trend except possibly that longer prothrombin clotting times are associated with lower concentrations of fibrinogen. This possibility was investigated by calculating the correlation coefficient which was found to be −0.145. Since this figure fails to exceed the tabular value of 0.3246 given by Fisher and Yates[9] for a probability of 0.05

which corresponds to 1 chance in 20, no correlation can be said to exist. Consequently, the prothrombin time in these data is independent of the amount of fibrinogen in the plasma. However, this does not exclude the possibility that in certain as yet unknown diseases a correlation between these two variables will be found.

SUMMARY AND CONCLUSIONS

No relationship was found to exist between the prothrombin clotting time and the concentration of fibrinogen in the plasma in normal subjects and in patients with a variety of gastrointestinal lesions.

REFERENCES

1. Quick, A. J.: The Nature of the Bleeding in Jaundice, J. A. M. A. **110**: 1658, 1938.
2. Page, R. C., and Russell, H. K.: Prothrombin Estimation Using Russell Viper Venom. I. Simple Modification of Quick's Method, J. Lab. & Clin. Med. **26**: 1366, 1941.
3. Page, R. C., de Beer, E. J., and Orr, M. L.: Prothrombin Studies Using Russell Viper Venom. II. Relation of Clotting Time to Prothrombin Concentration in Human Plasma, J. Lab. & Clin. Med. **27**: 197, 1941.
4. Page, R. C., de Beer, E. J., and Orr, M. L.: Prothrombin Studies Using Russell Viper Venom. III. Effect of Lecithinized Venom on Prothrombin Clotting Time, J. Lab. & Clin. Med. **27**: 830, 1942.
5. Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A.: Prothrombin Estimation: A Procedure and Clinical Interpretations, Proc. Soc. Exper. Biol. & Med. **50**: 85, 1942.
6. Howe, P. E.: Determination of Protein in Blood—A Micro Method, J. Biol. Chem. **49**: 109, 1921.
7. Wu, H.: A New Colorimetric Method for the Determination of Plasma Protein, J. Biol. Chem. **51**: 33, 1922.
8. Koch, S. C., and McMeekin, T. L.: A New Direct Nesslerization Micro-Kjeldahl Method and a Modification of the Nessler-Folin Reagent for Ammonia, J. Am. Chem. Soc. **46**: 2066, 1924.
9. Fisher, R. A., and Yates, F.: Statistical Tables, Edinburgh and London, 1938, Oliver and Boyd, Ltd.

PROTHROMBIN STUDIES USING RUSSELL VIPER VENOM

V. Interbatch Variations of Venom

Robert C. Page, M.D., and Edwin J. de Beer, Ph.D.
New York, N. Y.

Russell viper venom has been used for more than two years by us as a thromboplastic agent in a modified form of Quick's prothrombin test.[1] The modification consists in substituting Russell viper venom for brain thromboplastin. However, more information on the stability and uniformity of different batches of Russell viper venom is desirable when it is to be used in such a quantitative time relation test.

Other workers using Russell viper venom in this test have mentioned the subject of interbatch variations. Witts and Hobson[2] stated that different

From the Chemical Laboratory of the Department of Surgery, College of Physicians and Surgeons, Columbia University and Presbyterian Hospital, New York, and The Burroughs Wellcome & Co. (U. S. A.), Inc., Experimental Research Laboratories, Tuckahoe, N. Y.

Received for publication, July 23, 1942.

batches of Russell viper venom as purchased vary in coagulant power but that vials from the same batch are very constant. Crosbie and Scarborough[3] used two different batches of venom which did not differ appreciably in activity.

The object of the present study was twofold: (1) to learn how much variation existed between batches of Russell viper venom and (2) to determine if ageing had any effect on the potency of this type of preparation.

DESCRIPTION OF PREPARATION

The venom of the Russell viper (*Vipera russellii*) is collected by the usual methods employed at snake farms in India. The venom is then dried, sterilized, and standardized by comparing it with a sample of known potency on fowl and horse plasma. Following this the venom is packed in dry form in nitrogen-filled glass ampoules.

Venom in ampoules, of different batches, has been collected for the past four years and kept at room temperature so that when this study was begun samples from thirteen different batches were available and they varied in actual age up to five and one-half years.*

METHOD OF STUDY

Four essentially normal adults were selected, venous blood samples were obtained and prothrombin clotting times determined for each blood sample with each of the thirteen batches of venom.

The method employed was Quick's procedure[1] modified by using Russell viper venom instead of brain thromboplastin.[4, 5] Eighteen cubic centimeters of venous blood were drawn into a dry syringe and mixed with 40 mg. of potassium oxalate in a centrifuge tube. The blood was centrifuged at 1,500 r.p.m. for five minutes, the oxalated plasma was drawn off, and the tests were done immediately. The test on each batch was performed as follows: 0.2 c.c. of oxalated plasma was pipetted into a small test tube (75 by 10 mm.) and 0.2 c.c. of Russell viper venom,† 1:10,000, solution was added. Calcium chloride solution (1.11 Gm. calcium chloride per 100 c.c.), 0.2 c.c., was then added and the stop watch immediately started. The tube was agitated for from ten to fifteen seconds and tilted until separate discrete fibrin particles could be seen. The prothrombin clotting time in normal individuals using Russell viper venom has been found to be 20.76 seconds ± 2.32 seconds[5] and 19.5 seconds ± 2.9 seconds.[6] Each of the four subjects had essentially a normal prothrombin clotting time.

RESULTS OF STUDY

The results are given in Table I. In order to determine whether significant variations existed between batches and whether there was evidence of deterioration with age, the data were plotted as in Fig. 1. It will be observed that the mean prothrombin clotting times obtained with all batches regardless of age fell

*In the experiments, age of venom refers to elapsed time in days between the labeled "expiration date" and the date of the experiment. The venom actually was one year older than these figures indicate because the expiration date on the ampoule is one year in advance of the date on which the venom was placed in the ampoule.

†Supplied as "Stypven" Russell Viper Venom by The Burroughs Wellcome & Co. (U. S. A.), Inc., New York, N. Y.

TABLE I

DATA ON PROTHROMBIN CLOTTING TIMES OF FOUR ADULTS AS OBTAINED WITH VENOM OF THIRTEEN DIFFERENT BATCHES

VENOM BATCH NO.	AGE* IN DAYS	PROTHROMBIN CLOTTING TIME (SEC.)				MEAN BATCH PROTHROMBIN CLOTTING TIME (SEC.)
		PATIENT A	PATIENT B	PATIENT C	PATIENT D	
RV 30 C	1,632	20.0	19.5	23.2	23.7	21.6
RV 99 B	1,046	21.2	20.2	30.0	25.5	24.2
RV 122 D	981	22.0	20.8	24.0	23.8	22.6
RV 134 A	881	23.8	21.0	23.4	21.2	23.2
RV 146 A	731	21.7	21.6	27.5	21.2	23.0
RV 158 B	610	22.6	20.4	28.1	24.8	24.0
RV 164 A	566	23.5	21.7	23.0	24.0	23.0
RV 191 B	276	22.8	21.9	28.1	23.0	23.9
RV 199 D	177	23.8	23.1	28.4	24.2	24.8
RV 200 A	171	20.8	19.8	25.8	22.4	22.2
RV 210 A	37	23.8	21.1	21.7	21.9	22.1
RV 212 A	27	22.8	20.1	23.0	24.1	22.5
RV 230 B	-224	22.8	20.0	23.4	21.0	22.3

*See first footnote on p. 913.

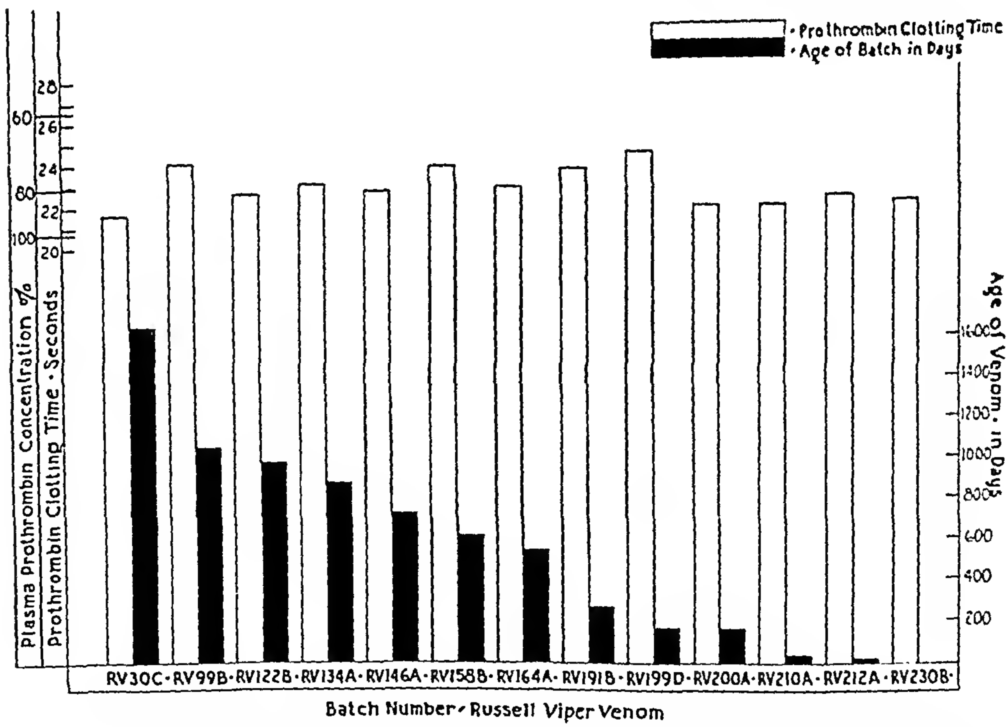


Fig. 1.—Bar graph showing the variations in prothrombin clotting time obtained with thirteen batches of Russell viper venom of different ages. Clear columns represent mean prothrombin clotting time; black columns represent age in days (time elapsed between expiration date and day of experiment) of the particular batch of venom.

within very narrow limits, roughly twenty-two to twenty-four seconds. Also, although the age of the oldest batch was 1,632 days (almost four and one-half years), its mean prothrombin time value (21.6 seconds) was almost the same as that obtained with the newest batch bearing an expiration date that would

not be reached until about eight months after this study and having a mean prothrombin clotting time of 22.3 seconds. It seems obvious, therefore, that as far as this study goes, age did not affect the potency of the preparation.

A statistical examination of the data, in which an analysis of variance was calculated, revealed that no significant variation between batches existed. F, the variance ratio, was 1.386 which, according to Snedecor's tables,[7] is not to be considered significant. If patients with prolonged prothrombin clotting times had been used in this study, greater variations would have been introduced, and the difficulty of detecting possible interbatch variations would have been increased.

SUMMARY AND CONCLUSIONS

1. Samples of Russell viper venom in nitrogen-filled ampoules were found to be stable over a period of at least five and one-half years.

2. Variations between thirteen different batches were not found to be significant.

We wish to express our appreciation for the assistance and cooperation of Dr. Louis Bauman in whose laboratory this work was carried out.

REFERENCES

1. Quick, A. J.: The Nature of the Bleeding in Jaundice, J. A. M. A. 110: 1658, 1938.
2. Witts, L. J., and Hobson, F. C. G.: Analysis of Haemorrhagic States With Snake Venom and Lecithin, Brit. M. J. 2: 862, 1940.
3. Crosbie, A., and Scarborough, H.: Activation of Thromboplastin Preparations by Haemolysis and by Lecithin, Brit. M. J. 1: 268, 1941.
4. Page, R. C., and Russell, H. K.: Prothrombin Estimation Using Russell Viper Venom, I. Simple Modification of Quick's Method, J. Lab. & Clin. Med. 26: 1366, 1941.
5. Page, R. C., de Beer, E. J., and Orr, M. L.: Prothrombin Studies Using Russell Viper Venom, II. Relation of Clotting Time to Prothrombin Concentration in Human Plasma, J. Lab. & Clin. Med. 27: 197, 1941.
6. Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A.: Prothrombin Estimation: A Procedure and Clinical Interpretations, Proc. Soc. Exper. Biol. & Med. 50: 85, 1942.
7. Snedecor, G. W.: "Statistical Methods," Ames, Iowa, 1938, Collegiate Press, Inc.

A SIMPLIFIED TECHNIQUE FOR MEASURING RENAL BLOOD FLOW AND TUBULAR EXCRETORY MASS

THOMAS FINDLEY, M.D., JOSEPH C. EDWARDS, M.D., ETTA CLINTON AND H. L. WHITE, M.D., ST. LOUIS, MO.

A RELIABLE simplification of Smith's original technique[1] for determining renal blood flow and tubular excretory mass would be highly desirable. We have previously reported[2] that the subcutaneous injection of diodrast and inulin can produce plasma concentrations suitable for clearance studies and that this method is very much more simple than that which depends upon constant intravenous infusion. Plasma iodine concentrations sufficiently high for the measurement of diodrast-Tm cannot be established in this way, however, so we have sought an empirical substitute. This report concerns an attempt to predict diodrast-Tm from the total quantity of diodrast excreted during the thirty minutes following a single intravenous injection of a standard dose. It appears that the percentage of diodrast excreted under these conditions is sufficiently proportional to diodrast-Tm to permit prediction of the latter from a line chart. The combination of these two modifications greatly simplifies the entire procedure and reduces the required number of blood and urine specimens by nearly one-half.

Plasma Diodrast and Inulin Clearance.—For subcutaneous use the 35 per cent diodrast solution* is irritating unless diluted about three times with saline. Inulin (Pfanstiehl, C. P.) must be thoroughly purified by boiling in water, filtering, and reprecipitating with alcohol three or four times until there is no visible residue on the filter paper; before each experiment the required amount is boiled again in 0.5 per cent saline sufficient to make a 25 per cent solution and injected while warm enough to remain in solution.

The optimal plasma concentration of diodrast is 1 to 5 mg. per 100 c.c.; of inulin, 5 to 15 mg. per 100 c.c. We originally reported that these may be achieved by the use of 0.2 c.c. diodrast and 0.2 Gm. of inulin per kilogram of body weight.[2] Extended experience has shown that amounts half this large are usually adequate, particularly in abnormal subjects. This means that a 70 kg. subject requires the subcutaneous injection of about 20 c.c. of diluted diodrast and 28 c.c. of 25 per cent inulin, but little discomfort arises since half of each solution is injected into the loose tissue of each axilla. Furthermore the injections of diodrast and inulin are separated by an interval of about forty-five minutes.

From the Departments of Medicine and Physiology, Washington University School of Medicine, and the St. Louis City Hospital, St. Louis, Mo.

Aided by Grants from the Smith, Kline and French Laboratories, Philadelphia (T. F.), and the Commonwealth Fund (H. L. W.).

Received for publication, July 27, 1942.

*Supplied by The Winthrop Chemical Co.

Diodrast-iodine was determined by the method of White and Rolf[3] and inulin by a slight modification of that of Corcoran and Page.[2]

Diodrast-Tm Substitute.—In a study concerned with intravenous pyelography,[4] we have shown that normal subjects who received an intravenous injection of diodrast (0.2 to 0.6 c.c. kg. at a uniform rate during a five-minute period) excrete a relatively constant fraction of this dose during the next half hour but that the plasma iodine concentration falls somewhat below that required for Tm determination at the end of this time. Since at high plasma iodine concentrations the glomerular and tubular contributions to total urinary diodrast are approximately equal and since our abnormal subjects[5] have shown no marked disproportion between glomerular filtration rate and tubular excretory mass, it seemed likely that reductions in diodrast-Tm would be reflected by proportionate reductions in total diodrast excretion rates.

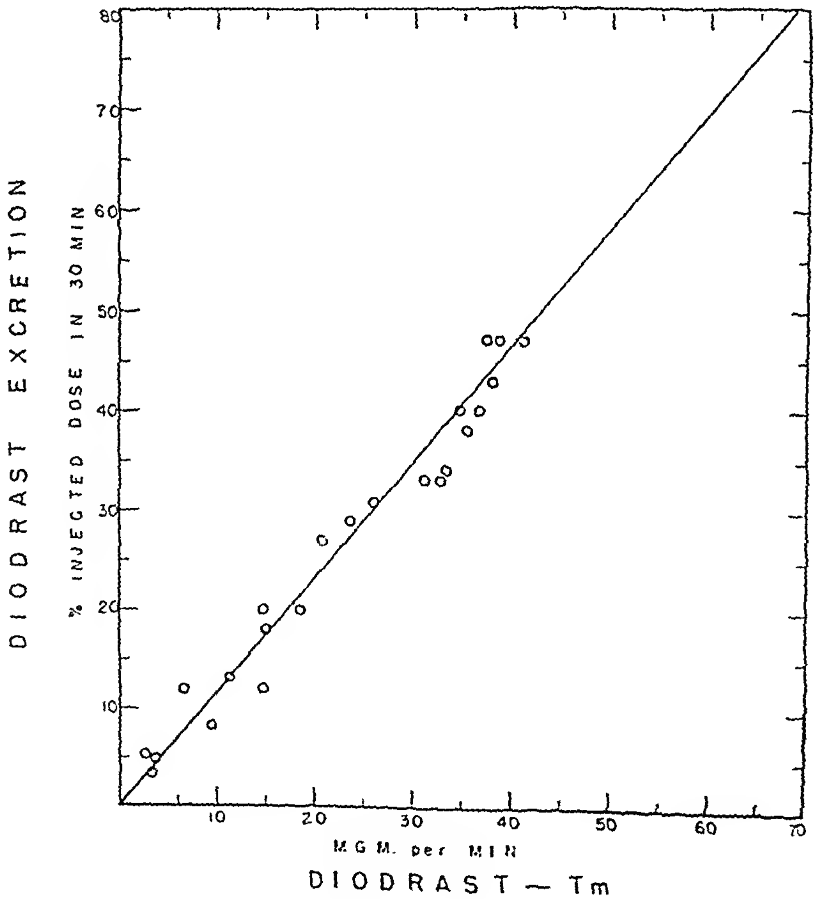


Chart 1.—Correlation between diodrast-Tm and the thirty-minute test.

Chart 1 shows results obtained on a series of normal subjects and patients with various renal disorders. The diodrast-Tm determinations were made by a slight modification of Smith's technique; most of them have been reported elsewhere.[5] Each point represents the average of at least three periods. Two or three days later each subject was given an intravenous injection of diodrast

(0.3 c.c. kg.) at a uniform rate during a five-minute period and the total amount excreted in the thirty minutes following completion of the injection determined. Expressed as a fraction of the injection dose, this value was plotted against diodrast-Tm. In Chart 1 it is not intended to minimize the occasional wide discrepancy at low Tm levels; the percentile difference is sometimes large but not, in our experience, larger than the fluctuations which have appeared in consecutive Tm periods obtained by the conventional infusion technique. This series is too small for statistical analysis, but it suggests that the error in predicting Tm from this line chart is less than ± 10 per cent.

Except for a few specially trained subjects, all of the half-hour urine collections were made by catheter and repeated bladder washings.

Combined Technique.—An indwelling catheter is applied to the fasting but hydrated subject, and a blood sample is obtained which serves as a blank for the diodrast and inulin determinations.

The subject lies on his back with his hands under his head. Under 1 per cent procaine anesthesia half of the inulin solution is injected into the subcutaneous tissues of each axilla at about the fifth interspace in the anterior axillary line. A 25 c.c. glass syringe is usually adequate and a three-inch 18-gauge needle is satisfactory. If the needle is fully inserted and slowly withdrawn during injection, no great rupture of tissue occurs. We have not massaged the areas after injection.

About forty-five minutes later the diluted diodrast is injected into the same places. This may cause momentary burning.

About fifteen minutes later the first blood sample (heparin) is obtained, the bladder is washed with saline, and clearance periods are established in the usual manner. Satisfactory plasma concentrations of diodrast and inulin exist for at least three hours.

The final blood sample is withdrawn after the last urine collection has been made. The quantity of undiluted diodrast necessary for Tm estimation (0.3 c.c. kg. in five minutes) is injected through the same needle and the bladder washed for the last time exactly thirty minutes after the end of the injection period. This sample is analyzed for diodrast only and completely replaces the minimum of three blood and three urine samples required by the intravenous technique.

It is of course essential that the catheter be draining freely and that the bladder be thoroughly washed. We have usually used five 20 c.c. portions of saline for this purpose, and all of these are added to the voided specimen before analysis.

One operator can easily examine two subjects simultaneously in this way.

DISCUSSION

Account must of course be taken of the diodrast contributed to the urine by the subcutaneous depots during the Tm period. The points in Chart 1 were obtained on diodrast-free subjects, but the combined technique described above requires that the thirty-minute test be performed on individuals whose

urine already contains diodrast coming from the axillary reservoirs. Failure to correct for the iodine from subcutaneous sources may increase the Tm estimate by 20 per cent.

Chart 2 presents the necessary correction factors; it is compiled from 195 plasma diodrast clearance periods in sixty-six subjects. Since the rate of diodrast excretion depends upon (a) the plasma concentration and (b) the plasma clearance, we have divided our renal blood flow data into five groups, depending upon the mean plasma diodrast concentration obtained during the clearance periods: 0 to 1, 1 to 2, 2 to 3, 3 to 4, and 4 to 5 mg. per 100 c.c. Each group is

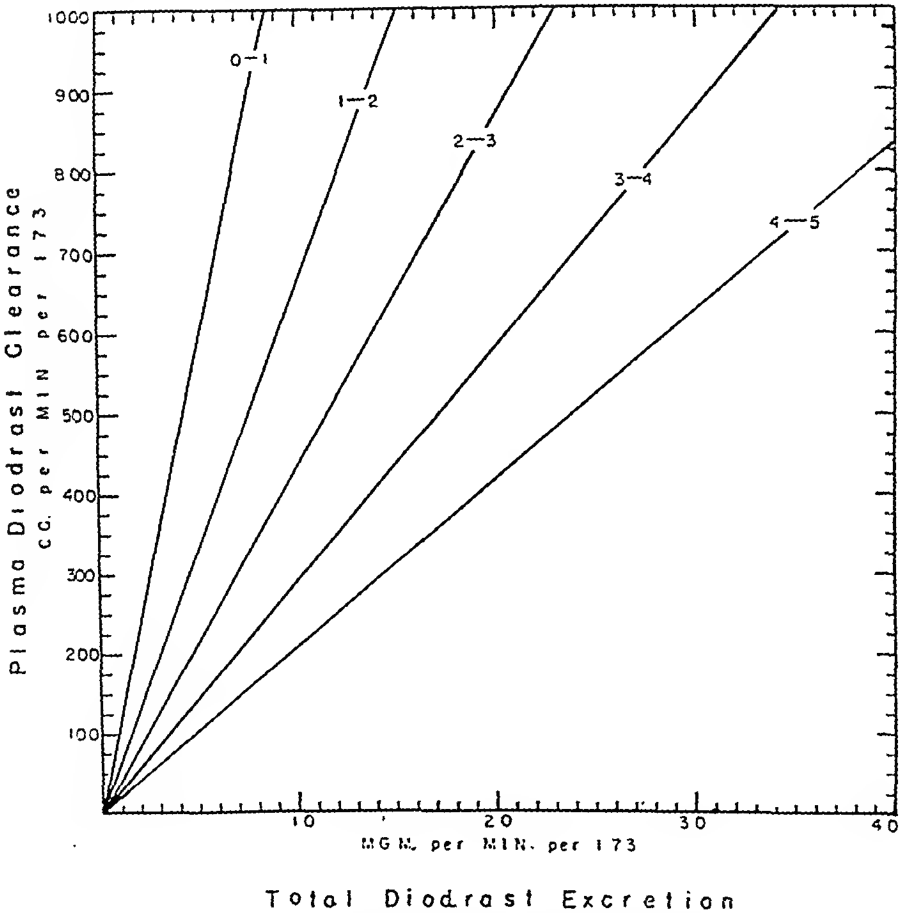


Chart 2.—Correction factors for diodrast-Tm prediction. The numbers on the slanting lines represent plasma diodrast-iodine concentrations in mg. per 100 c.c. From these values and the observed plasma clearances the quantity of diodrast coming from subcutaneous sources during the thirty-minute test may be predicted.

represented by a slanting line and contains plasma clearances plotted against total diodrast excretion rates. Assuming that the plasma clearance of subcutaneous diodrast is not altered by the intravenous injection, one may subtract the predicted total diodrast excretion rate for every minute elapsing between the last two bladder washings. This interval is, of course, variable, since it includes not only the five-minute injection period and the subsequent half-hour collection period, but also such time as may be necessary for obtaining the final

blood sample, changing syringes, etc. It has seemed better to make this correction than to attempt to wash the bladder twice in rapid succession. It is believed that these corrections for subcutaneous diodrast reduce possible errors in Tm estimation not dependent upon technical factors to considerably less than 5 per cent. These correction factors are probably a little too large, since renal diodrast extraction is more nearly complete at low than at high plasma concentrations.

CONCLUSION

A line chart is presented which permits prediction of diodrast-Tm from a single urine specimen. When this is combined with the subcutaneous method for determining renal plasma flow and glomerular filtration rate, the entire procedure is greatly simplified.

REFERENCES

1. Smith, H. W., Goldring, W., and Chasis, H.: The Measurement of the Tubular Excretory Mass, Effective Blood Flow and Filtration Rate in the Normal Human Kidney, J. Clin. Investigation 17: 263, 1938.
2. Findley, T., and White, H. L.: Measurement of Diodrast and Inulin Clearances in Man After Subcutaneous Administration, Proc. Soc. Exper. Biol. & Med. 45: 623, 1940.
3. White, H. L., and Rolf, D.: Modified Method for Determination of Certain Organic Iodine Compounds, Inorganic Iodide in Plasma and Urine, Proc. Soc. Exper. Biol. & Med. 45: 433, 1940.
4. Findley, T., Edwards, J. C., Clinton, E., and White, H. L.: Intravenous Urography: Test of Renal Function, J. Urol. 48: 119, 1942.
5. Findley, T., Edwards, J. C., Clinton, E., and White, H. L.: Clearance of Diodrast, Phenolsulfonthalein and Inulin in Hypertension and Nephritis, Arch. Int. Med. 70: 935, 1942.

MEDICAL ILLUSTRATION

COLOR PHOTOGRAPHS SIMPLIFIED*

Valentine J. Connolly, Staten Island, N. Y.

It is generally conceded that color photography is a valuable asset in the presentation of post-mortem material. We have evolved a setup which, we believe, reduces the problems of equipment, expense, personnel, and time to a minimum.

The only equipment required is a miniature camera and a homemade frame of ordinary wood. The color film used is the least expensive made and it costs but ten cents for each picture. The photographic work can be done by a member of the regular staff, thus eliminating the need of specially trained individuals. This is essential since most hospitals do not have sufficient photographic work to employ a full-time photographer. Once the setup is calibrated, it takes less than one minute to take a picture.

The apparatus is shown in Fig. 1. For the most part it is made of wood. The base should be of heavy material to secure immobility and minimize vibration. The overall dimensions are about thirty inches by thirty inches. The area upon which the specimens are placed to be photographed (the millimeter rule in Fig. 1) is eighteen inches square. It is so fashioned that it can be slid up or down upon wedges made for that purpose. The wedges are placed in steps about one and one-half inches apart. The camera is attached by a wooden extension and placed so that the lens is centered exactly in the middle of the plate upon which the specimens are to be placed. The camera must be fixed rigidly and left in the proper position. Two No. 2 photofloods in reflectors (cardboard reflectors would do) are attached to upright posts at either side. The lights are set equidistant from the center of the specimen plate to illuminate evenly the area to be photographed. When satisfactorily arranged they should not be disturbed.

In our particular setup a Kodak Bantam with an Ektar F :2 lens of 45 mm. focal length is used. Almost any miniature camera could be adapted to the purpose. A portrait lens placed in front of the regular lens serves the dual purpose of decreasing the working distance and more fully utilizing the film area. According to the portrait lens used, the size of the image on the film may be increased one to two diameters. This is advantageous because it results in a larger screen image when projected. A polarizing screen may be added to the system to reduce reflection and high lights. Once it has been set to polarize the permanently placed light sources, care should be taken that it is not moved from that position.

---

*From the U. S. Marine Hospital.

Received for publication, Feb. 16, 1943.

To calibrate the setup the back of the camera is opened and a piece of ground glass placed at the film plane. The maximum and minimum distances of focus can be determined by placing a millimeter ruler on the specimen board and raising and lowering the board by steps while observing the image on the ground glass. The highest and lowest satisfactory steps are marked and when a small specimen is photographed the highest step is used; the lowest step is used for the large specimens. The board is then placed halfway between these points and with the lens wide open a critically sharp focus is obtained of the millimeter divisions on the ground glass. When this setting has been chosen it should not be moved by jarring. The shutter is then closed and color film inserted. The shutter speed should be set at the longest available time, one second if possible.

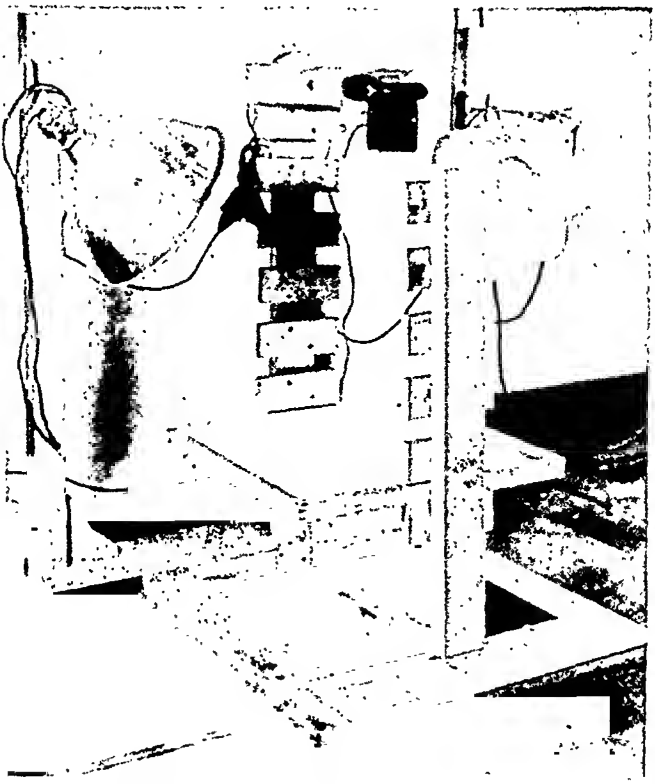


Fig. 1.—Photograph of apparatus showing fixed camera and lights and movable specimen board.

An average specimen, such as a heart, should be used for the calibration. The lens opening is closed to the lowest stop and an exposure made. The frame is moved along, the lens opening increased one stop, and another exposure taken. This procedure is repeated until all available lens openings have been used with the same shutter speed. The purpose of the slowest shutter speed is that the smallest lens opening will be utilized and consequently the greatest depth of focus will be obtained. After the films have been processed they may be projected to ascertain the best exposure. The lens stop is returned to this setting and kept there. The time setting remains stationary. To take subsequent pictures it is

merely necessary to place the specimen in the proper place and make the exposure. The only variable is the specimen.

The method is not without limitations. Exceptionally small and unusually large specimens cannot be reproduced well. Extremes in the color of some specimens, such as a dark blue lung or a white aortic vessel, will not be perfectly exposed at the medial exposure selected.

SUMMARY

A simplified method of taking color photographs of post-mortem specimens is presented. The necessity of taking light readings, focusing the specimen, and making the usual camera and light adjustments is eliminated. By reducing the variables to one (the specimen), excellent results are generally obtained.

BOOK NOTICES

Psychogenic Factors in Bronchial Asthma*

PSYCHOGENIC *Factors in Bronchial Asthma* consists of two volumes, the first a symposium by several members of The Institute for Psychoanalysis, Chicago, and the second a series of case reviews. Allergists, especially in the early days of this branch of medicine, were guilty of a one-sided outlook in their efforts to explain and cure all asthma entirely on the basis of skin tests. More recently their viewpoint has broadened so that good allergists today give as much attention to nonallergic factors such as focal infections and psychogenic problems, as they do to specific therapy. The reviewer's impression of the monograph under discussion is that the psychoanalyst, having discovered a psychogenic factor in some cases of asthma, has become guilty of the same hyperenthusiasm for his particular approach. The cases studied by the method of dream analysis are too few to be inclusively convincing. The observations are interesting and should be especially so to those allergists who may have devoted too little attention to psychogenic factors. One still wonders what happens to the asthmatic who doesn't dream.

Manual of Clinical Laboratory Methods†

A COMPREHENSIVE, technical, loose-leaf compendium of methods of interest to the technician.

Synopsis of Blood Diseases‡

THE purpose of this small book is to present in succinct form a synopsis of the salient features of diseases of the blood, addressed primarily to the senior student and the practitioner.

This is admittedly a difficult objective. The book is, hence, somewhat difficult to evaluate. It was once said that if the Tallqvist hemoglobin scale served no other purpose than to acquaint the observer with the appearance of drawn blood, a useful purpose was achieved. In like manner, if this book serves only to call attention to the complexities of diseases of the hematopoietic system, it may also serve to emphasize the difficulties embraced in their thorough understanding.

The Rat in Laboratory Investigation§

THIS book represents a compilation of some thirty different collaborators, all well-known workers in their respective fields. It covers everything about the rat, from love at first sight to psychoses and intestinal parasites in the oncoming generation. Every virtue, every weakness, and every peculiarity of the rat is fully and completely described. The anatomy,

*Psychosomatic Medicine Monograph IV. Psychogenic Factors in Bronchial Asthma. Part I. By Thomas M. French, M.D., and Franz Alexander, M.D. With the Collaboration of various contributors. Paper, 92 pages. Volume II, Nos. I and II. Paper, 236 pages. Published with the Sponsorship of the Committee on Problems of Neurotic Behavior, Division of Anthropology and Psychology, National Research Council, Washington, D. C., 1941.

†Manual of Clinical Laboratory Methods. Opal E. Heppler, Ph.D., M.D., Assistant Professor of Pathology, Northwestern University Medical School. Planographed, 159 pages. John S. Swift Co., Chicago.

‡Synopsis of Blood Diseases. A. Piney, M.D., M.R.C.P. Physician, St. Mary's Hospital for Women and Children, London; Director, Pathological Department, Royal Cancer Hospital, London. Cloth, 120 pages, 4 colored plates, $2.75. The Blakiston Company, Philadelphia, Pa.

§Edited by John Q. Griffith, Jr., M.D., Associate in Medicine, School of Medicine, University of Pennsylvania, and Edmond J. Farris, Ph.D., Executive Director and Associate in Anatomy, Wistar Institute of Anatomy and Biology. Cloth, 488 pages, 178 illustrations, $7.50. J. B. Lippincott Company, Philadelphia, Pa.

physiology, biochemistry, pathology, psychology, and the full usefulness (or the lack thereof) of the rat in laboratory or experimental work is described in minute detail. Special chapters are devoted to breeding, diet, the teeth, digestion, metabolism, the central nervous system, psychologic phenomena, the circulatory system, biologic assay, hematology, osteology, radiology, histology, parasites and special diseases of the rat. Of particular interest are special sections on the eye of the albino rat, the surgery of the rat, and, of especial usefulness, a very extensive chapter (by Dr. Harold G. O. Holck) on dosage of drugs for rats. This latter chapter lists therapeutic and fatal dosages (when given in various ways) for some five hundred different drugs and is followed by a bibliography of 447 references. The chapter on "Technics for the Investigation of Psychological Phenomena in the Rat" (by Dr. George L. Kreezer) is followed by 678 references.

Well printed and admirably illustrated on excellent paper, this book is destined to become a sort of universal encyclopedia and reference work for all those who breed or use rats in experimental or investigational fields.

Macleod's Physiology in Modern Medicine*

SINCE the death of Dr. MacLeod his textbook on physiology has been edited by Philip Bard, Professor of Physiology at Johns Hopkins University School of Medicine with the collaboration of nine outstanding physiologists and pharmacologists. This is the ninth edition, the second under the new editorship. The usual number of additions and alterations have been incorporated, necessitated by advancing knowledge in the field. The work is so widely known that it does not require elaborate review. The paper used is especially desirable. Having a very faint green tinge, it is more restful on the eyes than the more usual white paper. The bibliography appears at the back of the book. It contains an innovation in that at the top of each page there is an index file referring to the pages in the book where the references appear.

Diseases of Metabolism†

DUNCAN'S *Diseases of Metabolism* is a contribution by fifteen outstanding contributors, well qualified in their fields, edited by Duncan, who has also contributed several sections. The subject matter adheres strictly to the title. The first half of the book deals with the metabolism and physiology of carbohydrates, fats, proteins, minerals, water, and vitamins. Expressed this way, one might have the impression that the discussion is a rehash of information long since available. Quite on the contrary, it consists of a splendidly arranged review of the abundant recent developments in the field. The first half of the volume alone places it in the category of better than average medical texts. It correlates much that has heretofore been available mostly in scattered articles.

Metabolic disturbances discussed in detail include undernutrition, obesity, xanthomatoses, gout, hyperinsulinism, diabetes insipidus, melituria and diabetes mellitus. The only chapter which might well have received more extensive discussion is that on undernutrition.

---

*Macleod's Physiology in Modern Medicine. Edited by Philip Bard, Professor of Physiology, Johns Hopkins University School of Medicine. With the Collaboration of Henry C. Bazett, Professor of Physiology, University of Pennsylvania, George R. Cowgill, Associate Professor of Physiological Chemistry, Yale University School of Medicine; Howard J. Curtis, Instructor in Physiology, Johns Hopkins University School of Medicine; Harry Eagle, Passed Assistant Surgeon, United States Public Health Service and Lecturer in Medicine, Johns Hopkins University School of Medicine; Chalmers L. Gemmill, Associate in Physiology, Johns Hopkins University School of Medicine; Magnus I. Gregersen, Professor of Physiology, College of Physicians and Surgeons, Columbia University; Roy G. Hoskins, Director of Research, Memorial Foundation for Neuro-endocrine Research; Research Associate in Physiology, Harvard Medical School; J. M. D. Olmsted, Professor of Physiology, University of California; Carl F. Schmidt, Professor of Pharmacology, University of Pennsylvania. Ninth Edition. Cloth, 1256 pages. The C. V. Mosby Company, St. Louis, 1941.

†Diseases of Metabolism. Detailed Methods of Diagnosis and Treatment. A Text for the Practitioner. Edited by Garfield G. Duncan, M.D., Chief of Medical Service "B," Pennsylvania Hospital; Associate Professor of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania. Contributors: Walter Bauer, Abraham Cantarow, Garfield George Duncan, Ferdinand Fetter, Cyril Normal Hugh Long, Louis Harry Newburgh, Tom D. Spies, Hugh R. Butt, Tracy Donald Cuttle, Frank Alexander Evans, Friedrich Klemperer, Edward Halton Mason, John Punnett Peters, Leandro Maués Tocantns, Abraham White. Fully illustrated including 7 plates in color. Cloth, 985 pages. W. B. Saunders Company, Philadelphia and London, 1942.

The Avitaminoses*

NEW facts are evolving so rapidly in the study of vitamins that it was fortunate that Eddy and Dalldorf's *Avitaminoses* required a second edition after three years. Even so, the authors state that advances in our understanding of the vitamins necessitated a complete rewriting of most of the text. This volume will be of service to biochemists, clinicians and nutritionists alike. The first portion deals with the chemical nature of the vitamins. There follows a chapter on the functions of the vitamins in cellular oxidation which will enable the average reader to comprehend more clearly the action of these food components. The major portion of the volume is given to the description of the nature and function of each vitamin, the physiologic changes accompanying deficiency and the results of treatment. In an appendix, laboratory tests for vitamins are described and there is an excellent table of the vitamin content of most foods.

A Study of the Blood in Cancer†

IF ONE would read the last page of *The Blood in Cancer* first, one would be inclined not to read more. The observations are reminiscent of "Doctor" Abrams and Huckleberry Finn's reaction to the light of the moon.

Going backward in this volume, part III, describes many tests for cancer which have been recommended by various authors including urine tests, enzyme tests, and the like.

The major portion of the book deals with the author's studies of blood cytology in cancer. Dr. Gruner believes that unusually viscid blood without rouleaux but with tight cohesion of the red cells into islands with sharply defined contours and an early appearance of a dense fibrin network with granular matter in the meshes and actively moving leucocytes, reduction in the lymphocytes, irregular nuclear contours of the neutrophils, large polymorphous platelets, abnormality in the contour of the nucleus in the monocytes, and mitochondrial inclusions in the mononuclear leucocytes usually indicates malignancy.

*The Avitaminoses. The Chemical, Clinical and Pathological Aspects of the Vitamin Deficiency Diseases. By Walter H. Eddy, Ph.D., Professor of Physiological Chemistry, Teachers College, Columbia University; Director, Bureau of Foods and Sanitation, "Good House-[illegible]" and Gilbert Dalldorf, M.D., Pathologist to the Grasslands and Northern [illegible] Westchester County, N. Y. Second Edition. Cloth, 519 pages, $4.50. [illegible]ompany, Baltimore, 1941.

†A Study of the Blood in Cancer with Special Reference to the Needs of the Tumour Clinic. Fully illustrated, including graphs and coloured drawings. By O. Cameron Gruner, M.D. (Lond.). Author of "Biology of the Blood Cells" and "Studies in Puncture-Fluids." Cloth, 100 pages, $4.00. Renouf Publishing Company, Montreal, 1942.

The Journal of Laboratory and Clinical Medicine

VOL. 28 | MAY, 1943 | NO. 8

CLINICAL AND EXPERIMENTAL

BLOOD PRESSURE FLUCTUATIONS IN BRONCHIAL ASTHMA

I. CLINICAL OBSERVATIONS

HOWARD OSGOOD, M.D., BUFFALO, N. Y.

IN RECENT years there has been considerable discussion in medical literature on blood pressure in relation to bronchial asthma. This has been chiefly concerned with the question as to whether or not the mean systolic pressure shows a sustained rise or fall during an attack. It is not the purpose of the present paper to discuss this blood pressure change but rather to draw attention to certain fluctuations of the systolic pressure, which occur with each respiratory cycle during the asthmatic paroxysm, and to discuss their significance.

Minor fluctuations of the systolic blood pressure, synchronous with respiration, occur uniformly in normal individuals, the high point coming during expiration, the low point during inspiration in quiet breathing.

The respiratory fluctuations which occur in patients suffering from an attack of bronchial asthma are of greater amplitude. When the asthmatic paroxysm is severe, these fluctuations may be of sufficient magnitude to produce a noticeable waxing and waning of the radial pulse to palpation. A more common finding, however, is a lesser degree of fluctuation, not appreciable at the wrist, but only noted when the blood pressure is taken by a sphygmomanometer. The failure of these fluctuations to be detected ordinarily in the radial pulse may explain why they are seldom mentioned in medical texts dealing with allergic conditions or in general medical texts in connection with bronchial asthma. Physicians whose practice entails the observation of many asthmatic patients have undoubtedly noted them repeatedly.

From the Medical Services and Allergy Clinic of the Buffalo General Hospital, Buffalo, N. Y.

Received for publication, Sept. 8, 1942.

Sihle,[1] in 1903, stated that a majority of asthmatic patients during a paroxysm show a variable lowering of blood pressure, "so that in the same patient, in the course of five minutes, not infrequently values of 90, 70, 100, 85, 75 can be observed." He did not note that these were related to the respiratory cycle.

Tinel and Jacquelin[2] studied a number of asthmatic patients by means of a cuff over the humeral artery and kymograph tracings of the pulse. They noted the changes produced in the tracings by varying the pressure in the cuff. They found (1) that during the attack of asthma, coincident with inspiration, there was a considerable diminution of the amplitude of the pulse tracings, which was absolutely constant during the course of each paroxysm, and which ceased with its termination; (2) that there was a fall of the "maximum arterial tension" at the onset of dyspnea, amounting to 20 mm. or 30 mm. Hg, with a return to the former level with the end of the paroxysm; and (3) that an *elevation* of the "minimum arterial tension" occurred at the onset of the paroxysm, resulting in a diminution of the oscillometric amplitude of the pulse. They concluded that these changes were due to some interference of the blood flow into the right heart and through the pulmonary circulation.

Rist, who discussed Tinel and Jacquelin's[2] paper, used the same method to determine the fluctuations of blood pressure which I have employed. He found that the systolic pressure (in various conditions) may vary as much as 30 *mm. Hg* between inspiration and expiration and maintained that the inspiratory suppression of the pulse noted by Tinel and Jacquelin was due to a lowering of the systolic pressure, and "was only an exaggeration of a normal phenomenon."

Rackemann[3] states that the respiratory fluctuations of the systolic blood pressure in asthma may be wide and are roughly parallel with the severity of the asthma. He also notes that there may be well-marked fluctuations in the presence of emphysema and many wheezes, when the severity of the asthma is not marked.

Feinberg[4] observes that during the asthmatic paroxysm, as a rule, the blood pressure rises and varies markedly with inspiration and expiration.

Detailed studies of the respiratory blood pressure fluctuations in bronchial asthma have not, so far as I am aware, appeared in the literature, and for this reason I present my findings.

METHOD

With practice, fluctuations of the systolic blood pressure in human beings, synchronous with the respiratory cycle, may be readily observed and measured by the auscultatory method, the stethoscope being placed over the antecubital space.

The usual 13 cm. cuff is applied to the upper arm and the sphygmomanometer placed close to the patient, so that it may be read and at the same time the patient's respirations can be seen "out of the *corner* of the eye." The cuff is inflated to a pressure above the highest systolic reading. The pressure is then slowly lowered, not more than 2 or 3 mm. per breath, the respiratory movements of the chest or abdomen being closely noted meanwhile. A point in the pressure will presently be reached, where a few beats will be heard at the elbow

at a certain moment in expiration. If the cuff pressure is held at this point, the auscultatory beats will disappear during the remainder of expiration and during the succeeding inspiration. There will be a trough of silence lasting until the corresponding moment in the next expiration, when the beats will reappear. The reading of the manometer at this point will indicate the highest level reached by the systolic pressure during the respiratory cycle.

As the cuff pressure is then further lowered slowly, more and more beats will be heard, first throughout expiration and then during inspiration. The manometer reading at the moment when beats are just heard throughout both inspiration and expiration marks the lowest point to which the systolic pressure falls during respiration. The difference between these two readings denotes in mm. Hg the amplitude of the respiratory fluctuations of the systolic pressure. The diastolic reading is taken in the usual way.

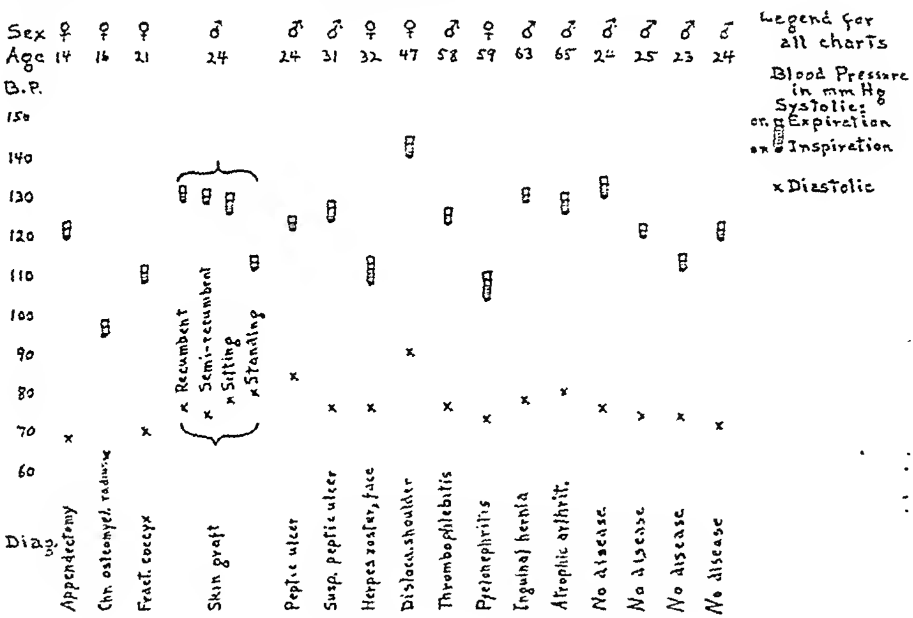


Fig. 1.—Normal respiratory fluctuations of the systolic blood pressure.

In my observations on asthmatic patients, and on normal individuals at rest, the high point of the systolic fluctuation has occurred uniformly during expiration and the low point during inspiration. It is possible that there may be circumstances where there is a reversal in phase of the high and low points, but I have not met with it. The diastolic pressure varies very slightly with respiration in asthma, but I have not included this variation in my studies. It is worth further attention.

In order to determine the normal limits of the respiratory fluctuation with this technique, determinations have been made on a number of subjects, both on healthy persons and on nonasthmatic hospital patients who were not acutely ill and were not suffering from cardiac, pulmonary, renal, or intrathoracic disease.

Fig. 1 shows the readings on sixteen such persons. The maximum fluctuation of the systolic pressure during respiration is 6 mm. Hg: the minimum is

2 mm. The average for the ten males is 3 mm.; for the six females it is 4 mm. Random observations on other similar individuals have shown an occasional fluctuation up to 12 mm. Hg in the absence of known disease.

OBSERVATIONS ON ASTHMATIC PATIENTS

Over the past three years determinations of the respiratory systolic fluctuation have been made on many asthmatic patients. The findings on some of these patients are here presented, with the high and low points of the systolic fluctuation indicated on the charts. The estimated severity of the asthmatic dyspnea at each reading is shown by the black columns at the bottom of the figures, with the dates beneath.

Fig. 2 shows findings on two patients on different days, the first reading in each being taken during an asthmatic paroxysm, the subsequent readings made when the paroxysm had subsided. In both, the fluctuation was wide when dyspnea was pronounced, diminishing when the dyspnea decreased.

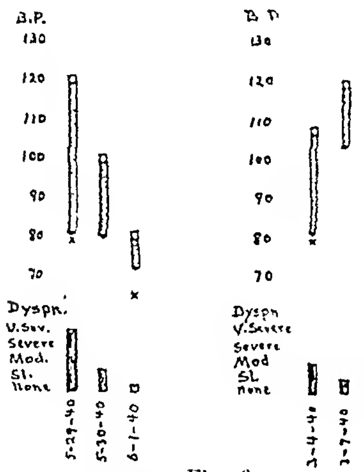


Fig. 2.

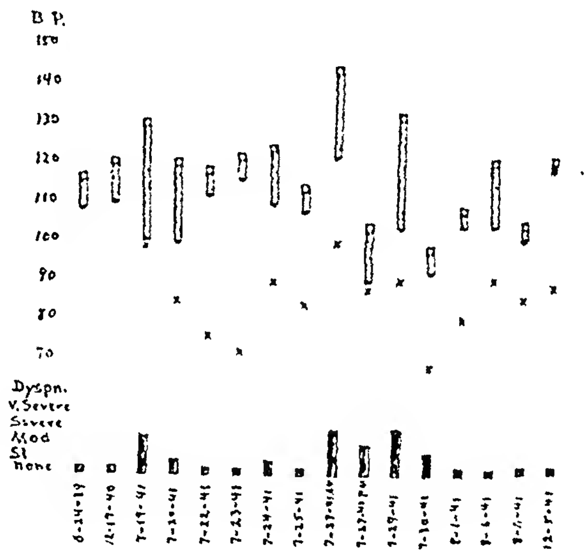


Fig. 3.

Fig. 2.—Respiratory systolic fluctuations at height of asthmatic paroxysm and after its subsidence. Patient B. B., female, 36 years of age, sensitive to inhalants and foods, and patient F. G., female, 38 years of age, sensitive to inhalants.

Fig. 3.—Respiratory systolic fluctuations in a chronic asthmatic patient, L. B., female, 48 years of age, with extrinsic and intrinsic factors as causative agents.

Fig. 3 presents readings taken at intervals over a period of two and one-half years on a chronic asthmatic patient whose asthma was due to intrinsic as well as inhalant factors. It will be noted that the respiratory systolic fluctuation followed closely the severity of the asthma, although on a few occasions it did not decrease to normal limits even though the patient was not apparently in distress. This corresponds to Rackemann's observation. One reading (third from the left) shows a fluctuation of 30 mm., the systolic pressure falling during inspiration almost to the diastolic level. This phenomenon has been noted in other patients when the asthma has been very severe.

Fig. 4 shows blood pressure readings taken over a period of a month (July 7 to August 7) on a patient 52 years of age who was very sensitive to grass and

weed pollens and to certain inhalants. During this month she passed through a very severe attack of asthma, being in an oxygen tent for five days. The respiratory systolic fluctuations parallel the severity of her asthma. Between July 20 and 29 there was a remission, at which time the fluctuations decreased to normal or near normal limits. A short recrudescence of the asthma then occurred with a corresponding increase in the systolic fluctuations, at one reading amounting to 30 mm. On August 7 she was asthma-free, and the fluctuation was 4 mm. Two readings in November, during a shorter attack of asthma, again disclosed an increase in the fluctuation. In this patient the mean systolic level tended to rise during a spell of asthma.

The determination of the respiratory systolic fluctuation has been useful in estimating the degree of bronchial obstruction present in a given patient at a given moment, as demonstrated in the following observations:

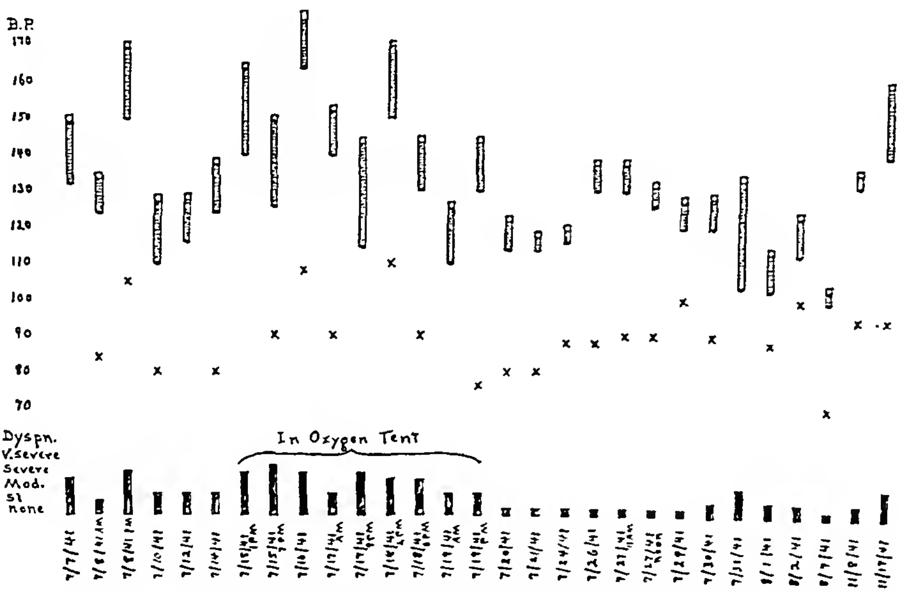


Fig. 4.—Respiratory systolic fluctuations throughout a prolonged attack of asthma. Patient G. H., female, 58 years of age, sensitive to pollens and inhalants.

An unmarried woman (Fig. 5), 53 years of age, was sensitive to orris, house dust, and grass pollen. She had had a number of moderately severe attacks of asthma and was highly apprehensive of its recurrence. A blood pressure reading during an asthma-free period yielded a respiratory systolic fluctuation of 5 mm. Three days later she complained of respiratory distress and a paroxysmal cough. When seen she was greatly excited and apprehensive, but to close observation she did not appear to be having much respiratory difficulty; an examination of her lungs showed only a few signs of bronchial spasm. The respiratory systolic fluctuation was 10 mm. With assurance and while preparations were being made to administer epinephrine in oil (but before it had been injected), her cough and distress subsided greatly. Ten minutes after the injection the respiratory systolic fluctuation was 7 mm. Here the distress was largely mental, and the blood pressure readings bore out the clinical impression that

there was little bronchial obstruction present. A stiff dose of sedative might have given as much relief as epinephrine, perhaps even more.

That relief of the bronchial asthma during an attack will bring about a reduction of the respiratory systolic fluctuation to within normal limits is demonstrated in Fig. 6. The patient was a married woman, 41 years of age who was primarily sensitive to orris root, with lesser sensitivity to house dust and flour mill dust. She had recurring attacks of severe asthma, but had developed no complicating conditions. Two blood pressure readings on December 2 and 4, when she was asthma-free, had shown respiratory systolic fluctuations of 3 and 4 mm., respectively. She was seen at 4 P.M. on January 13 in the midst of a

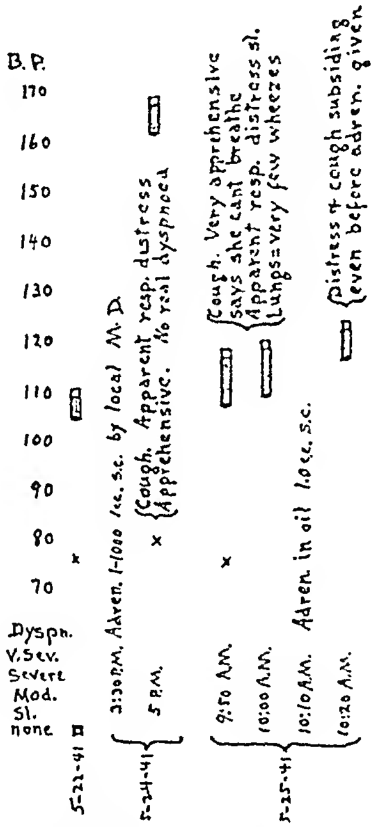


Fig. 5.—Respiratory systolic fluctuations in apparent asthmatic dyspnea but with little bronchial obstruction. Patient E. B., female, 53 years of age, sensitive to orris, dust and grass pollen.

severe attack; her lungs were full of wheezes. The respiratory systolic fluctuation was 30 mm. Injections of 0.25 c.c. epinephrine, 1 to 1,000, and 1 c.c. epinephrine in oil, 1 to 500, were given at 4:10 P.M. Some relief was noted at 4:20 P.M. and by 4:35 P.M. she was free of dyspnea, and the wheezes had disappeared from her lungs. Readings of the respiratory systolic fluctuation showed a progressive decrease in amplitude, until at 4:35 P.M. the fluctuation was 6 mm. A subsequent reading three months later, when she had no asthma, showed a fluctuation of 3 mm.

When adequate relief is not secured after palliative treatment, the continued dyspnea is reflected in a persistent wide respiratory systolic fluctuation.

In Fig. 7 are presented the findings in such an instance. The patient was a trained nurse, 43 years of age. She suffered from asthma at intervals throughout the year, with marked exacerbations during the grass season. Skin tests were negative except for a delayed positive to ragweed. She was seen on June 30 while in a severe asthmatic attack. The respiratory systolic fluctuation on two readings, taken ten minutes apart, were 26 and 45 mm., respectively. Injection of 0.6 c.c. epinephrine, 1 to 1,000, and 1 c.c. epinephrine in oil, 1 to 500, were then given. Five minutes after the injection she reported slight relief, and her respiratory systolic fluctuation had decreased to 16 mm. However, ten minutes later the relief had not been maintained and the fluctuation was 30 mm.

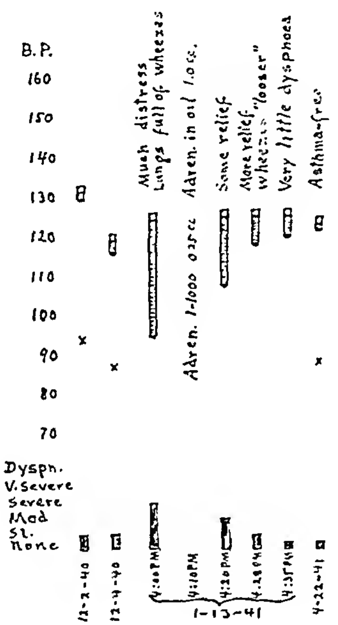


Fig. 6.

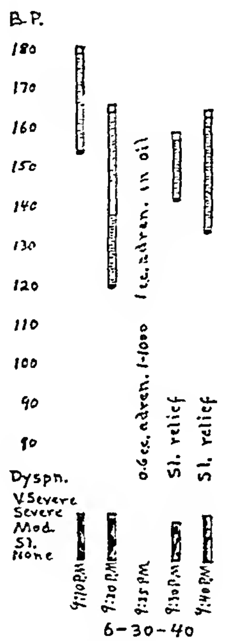


Fig. 7.

Fig. 6.—Respiratory systolic fluctuations before and after relief of an asthmatic paroxysm. Patient T. W., female, 41 years of age, sensitive to orris and other inhalants.

Fig. 7.—Respiratory systolic fluctuations when relief is not afforded by treatment. Patient M. M., female, 43 years of age, sensitive to pollens and inhalants.

DISCUSSION

The increased respiratory fluctuations of the systolic blood pressure reported above have been a constant finding in bronchial asthma.

A similar fluctuation (sharp fall in the systolic blood pressure) can be produced momentarily by attempting a deep inspiration with the nose and glottis closed (Mueller's experiment cited by Tinel and Jacquelin[2]). Certain experiments on cats, reported by me,[5] have shown fluctuations of the carotid blood pressure, similar to those in asthma, when obstruction to respiration was introduced at the trachea. Other experiments with increasing respiratory obstruction at the mouth in healthy young men, induced by progressively constricting a rubber mouthpiece, reported by me in the same paper,[5] also demonstrated an increasing fluctuation of the systolic blood pressure. In both cats and men, the amplitude of the fluctuations paralleled the degree of obstruction; and the

timing of the high and low points of the fluctuation in the respiratory cycle was the same as I have found it in asthmatic patients.

The mechanism by which this fluctuation is produced is probably complex. Reid,[6] in a recent article, has made some very interesting remarks on respiration as a factor in maintaining the circulation, which may throw light on the subject. The gist of his remarks is that during inspiration the intrathoracic pressure is lowered, favoring an inflow of blood from the large systemic veins into the chest, while at the same time the descent of the diaphragm raises the intra-abdominal pressure and so propels the blood through the vena cava into the large thoracic veins and to the right side of the heart. Also with the expansion of the lungs, the smaller pulmonary vessels dilate, so that a definite proportion of the whole blood volume is temporarily pooled in the vascular bed of the lungs. With the succeeding expiration, this pooled blood, plus the normal flow, is actively expelled from the lungs toward the left side of the heart by the elastic contraction of the lung tissue toward the hilum.

In bronchial asthma the intrathoracic and intra-abdominal pressure changes would be exaggerated. On *inspiration* it is possible that under the greater negative intrathoracic pressure[7] the vascular bed of the lungs dilates more than normal, pooling a greater amount of blood and withholding it from the left side of the heart. This would reduce the left ventricular output and so lower the systemic systolic pressure.

On *expiration*, which is considerably prolonged in bronchial asthma, there is an increased rise in the intrapleural pressure to even slightly above the atmospheric level.[7] This along with the elastic recoil of the lungs would induce an expulsion of the larger pool of blood in the lung-bed toward the left side of the heart, and a considerable increase of the left ventricular output would result, with a consequent rise in the systemic systolic blood pressure. Enhancing this greater supply of blood to the left side of the heart a moment later, but still during expiration, would be a greater supply of blood to the right side of the heart, as the increased intra-abdominal pressure, incident to the intense expiratory effort, propels more blood upward through the great veins into the chest. Since the intrathoracic pressure does not rise far above atmospheric pressure, whereas the intra-abdominal pressure starts at atmospheric level and is then increased by the vigorous action of the abdominal muscles in forced expiration, it is probable that this heightened pressure transmitted to the column of blood filling the vena cava would counteract any tendency of the positive pressure in the chest to collapse the veins entering the right side of the heart.

Whatever the true explanation, it is evident that the marked drop and subsequent rise of the systemic systolic blood pressure during the respiratory cycle in bronchial asthma is dependent upon some disturbance of the normal flow of blood through the lungs to the left auricle. This disturbance in turn is caused by the excessive fluctuations of the intrathoracic pressure induced by the obstruction to respiration in the smaller bronchi and bronchioles.

The parallelism between the presence and amplitude of the respiratory systolic blood pressure fluctuation on the one hand, and the severity of the asthma on the other, has been close, though not to a mathematical degree. I be-

lieve it may therefore be taken as an indirect and approximate measure of the degree of bronchial spasm or obstruction present and so may be a useful clinical sign. Conversely observations on the behavior of these fluctuations under various circumstances in bronchial asthma may offer a new method for studying the mechanism of the bronchial obstruction in this condition.

A paper is now in preparation covering observations on the effects of the administration of epinephrine and theophyllin ethylenediamine (aminophyllin) on these blood pressure fluctuations in asthmatic patients.

CONCLUSIONS

1. Wide fluctuations of the systolic blood pressure, synchronous with respiration, are a constant finding in bronchial asthma. The high point of the fluctuation occurs during expiration, the low point during inspiration.

2. The amplitude of these fluctuations parallels closely the severity of the asthma.

3. When the asthma has subsided, the fluctuations return to normal.

4. This respiratory systolic fluctuation is a useful clinical sign, indicating approximately the degree of respiratory obstruction present.

5. Observations on these fluctuations may be of value in studying the mechanism of bronchial asthma.

I wish to express my appreciation to Dr. Francis E. Ehret for his labor in going over innumerable records of asthmatic patients and abstracting the data on which this paper is based.

REFERENCES

1. Sihle, M.: Zur Pathologie und Therapie des Asthma, Wien. klin. Wchnschr. 16: 85, 1903.
2. Tinel, J., and Jacquelin, A.: Les modifications circulatoires peripheriques au cours de L'acces d'asthme, Bull. et mém. Soc. méd. d. hôp. de Paris 47: 440, 1931.
3. Rackemann, F. M.: Clinical Allergy, New York, 1931, The Macmillan Co., p. 431.
4. Feinberg, S. M.: Allergy in General Practice, Philadelphia, 1934, Lea & Febiger, p. 99.
5. Osgood, H., et al.: Blood Pressure Fluctuations in Respiratory Obstruction, Experimental Observations, J. Lab. & Clin. Med. 27: 1536, 1942.
6. Reid, W. D.: Respiration as a Factor in the Circulation of the Blood, Ann. Int. Med. 17: 206, 1942.
7. Prinzmetal, M.: Relation of Inspiratory Distention of the Lungs to Emphysema, J. Allergy 5: 493, 1934.

U-WAVE PATTERNS IN THE ABNORMAL ELECTROCARDIOGRAM*

SYLVAN D. SOLARZ,† M.D., AND STEPHEN R. ELEK,‡ M.D., CHICAGO, ILL.

THERE has been a dearth of literature on the U wave of the electrocardiogram and little is known concerning its cause and significance. The literature has recently been reviewed by Papp[1] who defined the dimensions of this wave and stressed the frequency of inverted U waves in clinical heart disease. Nahum and Hoff[2] presented evidence from which they concluded that the U wave is related to the supernormal phase of the myocardium and that it marks the period when premature ventricular systoles occur most frequently. Recent studies, including those from this laboratory,[3, 4] showing that many abnormal electrocardiograms fall into definite patterns, suggested the desirability of determining the U-wave contours occurring in these various patterns.

The electrocardiograms were selected at random from the files of the heart station according to the electrocardiographic patterns. Most of the records had chest leads CF_2 and CF_4 as well as the limb leads. A few had only one chest lead (CF_2) and a few had no chest leads. Serial tracings, especially in the cases of myocardial infarcts, were available in many instances.

Three chief electrocardiographic patterns were analyzed: (a) ventricular preponderance (425 cases), diagnosed according to criteria established in this department;[3-5] (b) myocardial infarction (350 cases); and (c) intraventricular block (QRS duration over 0.12 sec.) (210 cases). A small series of cases of pericarditis (6 cases), of acute glomerulonephritis (3 cases) and of pulmonary embolism (6 cases) were also studied.

CRITERIA FOR THE NORMAL U WAVE

Optimal heart rates for the identification of the U wave are listed by Papp[1] as 60 to 110 beats per minute. Below this rate, the U wave tends to fuse with the T; above this rate it tends to be incorporated with the P wave. In the course of this study a number of records were found in which normal or abnormally tall, upright U waves followed so closely after the T wave as to simulate notched, diphasic, or bizarre T waves (Fig. 1-*A*, *B*). These can be distinguished by measuring the QRST interval of the other leads. In cases of tachycardia examples of amalgamation of U and P waves occurred (Figs. 2, 5-*A*). Here the distinction can be made by measuring the P-R interval of the other leads. Furthermore, in records of auricular fibrillation, with slow or moder-

*From the Cardiovascular Department, Michael Reese Hospital, Chicago, Ill.
Aided by the A. D. Nast and Emily and Fanny Wedeles Funds for Cardiovascular Research.
Received for publication, Sept. 18, 1942.
†Sunset Camp Fellow. Now First Lieutenant, U. S. Army Air Force Medical Reserve.
‡Now First Lieutenant, U. S. Army Medical Reserve.

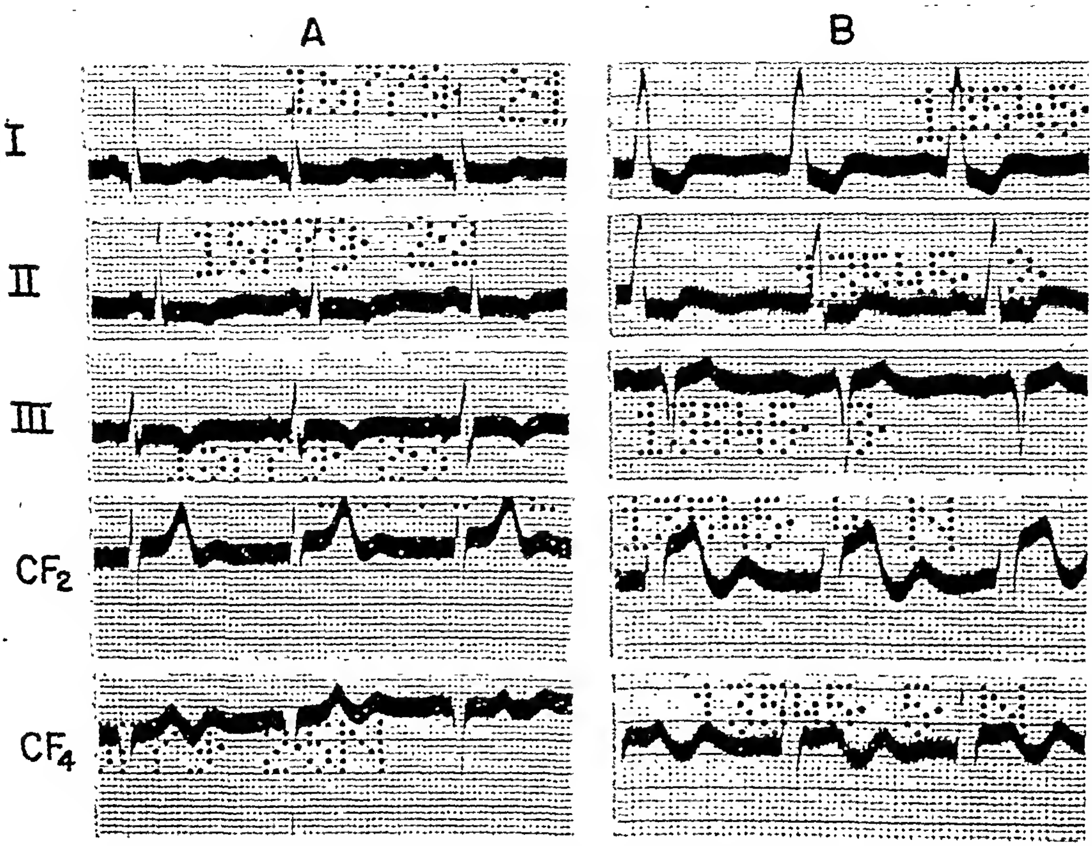


Fig. 1.—Simulated bizarre T waves caused by large U waves following closely after the T waves. *A.* In Leads I, II, CF2 and CF4 normal, upright U waves are seen giving the false impression of polyphasic T waves. *B,* Abnormally tall, upright U waves in the chest leads result in a configuration superficially resembling bizarre T waves.

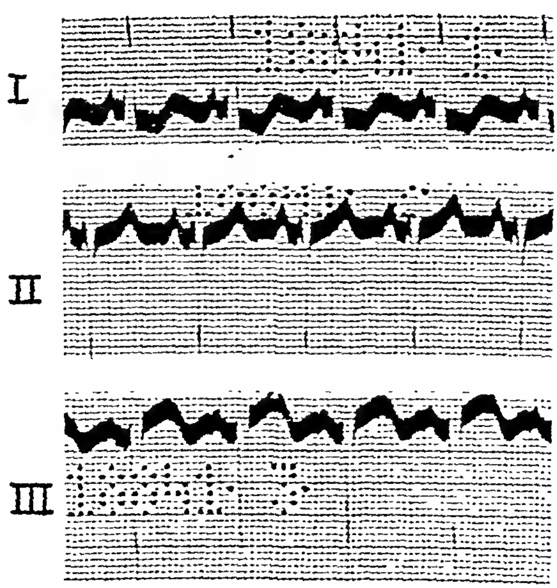


Fig. [illegible] the T and P waves in Lead III. This resembles a notched P wave. [illegible] in all three leads are within normal limits and the P wave in Lead [illegible] omparing the P-R intervals in the several leads.

ately fast ventricular rates, large upright U waves may exist, especially in the chest leads, lending the illusion of P waves (Fig. 3). These observations indicate that the recognition of even the normal U wave is important in avoiding errors in description of the P and T waves and in measurement of the P-R interval. The error in diagnosis of mistaking a U for a P wave in cases of auricular fibrillation is, of course, evident. In addition, the presence of the U wave means that the isoelectric level can best be determined in the interval between U and P.

The criteria of Papp[1] for normality of the U wave were accepted in our study. The normal U wave is upright, varying in height from 0.25 to 2.0 mm.

I

II

III

CF_2

CF_4

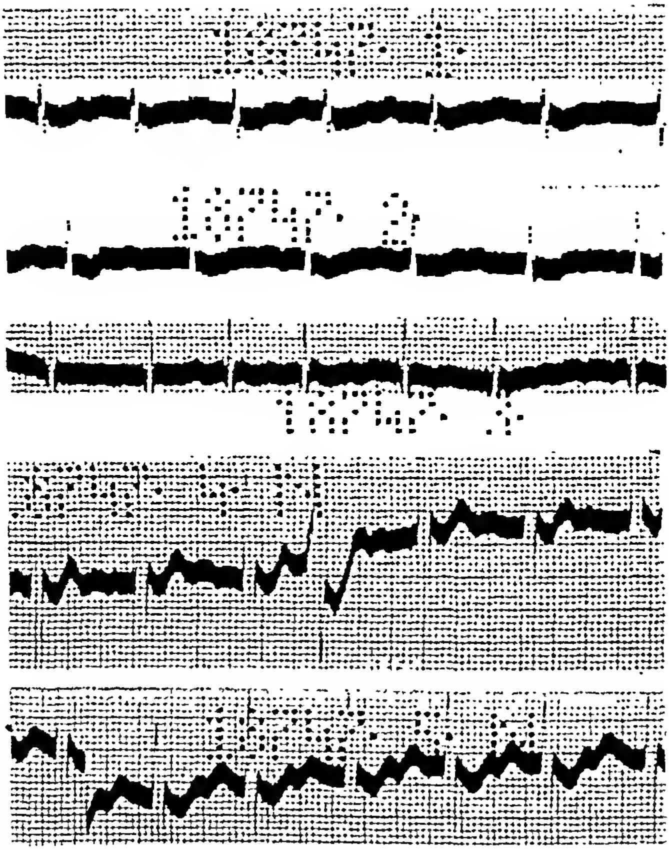


Fig. 3.—Auricular fibrillation of the coarse type. The U waves in leads CF_2 and CF_4 superficially give the impression of large P waves and might lead to an erroneous diagnosis.

I

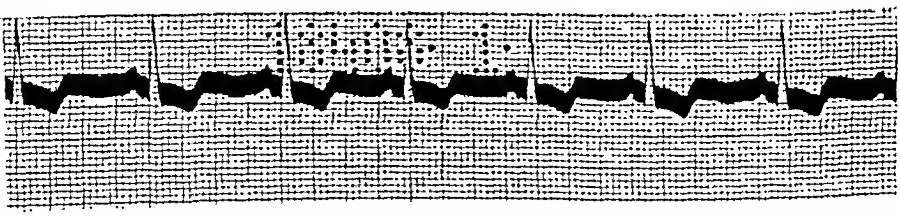


Fig. 4.—The downward bowed T-P segment in Lead I which is equivalent to an inverted U wave. Discussed in text.

and in duration from 0.18 to 0.24 sec. The U wave was considered abnormal when inverted, diphasic, or upright and taller than 2 mm. Another abnormality found by us especially in Lead I was the occurrence of a negative bowing of 1 mm. or more in the T-P segment of the electrocardiogram. In several records with this phenomenon the presence of a marked sinus arrhythmia soon made it evident that this bowing was due to the existence of an inverted U wave, for in the long T-P intervals the negative U wave was apparent; in the short T-P intervals only the downward bowing remained, the end of the U wave being invisible (Fig. 4). Hence this downward bowing of the T-P segment is regarded as an inverted U wave. A similar but normal upward bowing of the T-P segment may also be found.

THE U-WAVE PATTERNS IN HEART STRAIN

An analysis of 75 consecutive cases of right ventricular preponderance showed no abnormal U waves. Hence, the analysis of this pattern was not pursued further.

Examination of 350 cases of various types of left ventricular preponderance revealed 38 cases with abnormal U waves. Three cases were of the first type, 23 of the second type, 11 of the mixed type, and one of the concordant type.[4, 5]

The U-wave abnormality in the 3 cases of the first type of left ventricular preponderance consisted of U-wave inversion in CF_4 and on 2 occasions in Lead I. One instance of the inverted and one of the diphasic U were encountered in CF_2.

TABLE I

U-WAVE CONFIGURATION IN LEFT VENTRICULAR PREPONDERANCE OF THE SECOND TYPE IN THE 23 CASES SHOWING U-WAVE ABNORMALITIES

LEAD	U INVERTED	U UPRIGHT AND NORMAL	U UPRIGHT AND ABNORMAL	U DIPHASIC 1ST PHASE UP	U DIPHASIC 1ST PHASE DOWN	U ISO-ELECTRIC
I	17	1	0	0	0	5
II	2	5	0	0	0	16
III	0	11	0	0	0	12
CF_2	3	13	1	2	0	4
CF_4	11	7	1	0	1	2*

*One record had no CF_4.

Table I summarizes the configuration of the U wave in the second type of left ventricular preponderance. U-wave inversion was most frequent in Lead I, occurred rarely in Lead II and was not encountered in Lead III. It occurred more frequently in CF_4 than in CF_2. In addition, in the chest leads abnormal upright and diphasic U waves were found. It would appear, therefore, that the idealized pattern of the U wave in this type of left ventricular preponderance consists of an inverted U_1, an upright or isoelectric U_2 and U_3, an upright U in CF_2 and an inverted U in CF_4 (Fig. 5-*A*). The lead distribution of the abnormal U waves in this pattern is shown in Table VII. It is evident from this table that U-wave abnormalities occurred most frequently in Lead I and next in frequency in Lead CF_4.

A similar trend is revealed in the mixed type of left ventricular preponderance (Tables II and VII). In the case of the concordant type of left heart strain the U was found to be inverted in Lead I.

THE U-WAVE PATTERNS IN INTRAVENTRICULAR BLOCK

An analysis of 75 consecutive cases of intraventricular block of the S type[5] revealed no abnormal U waves. Hence, no further analysis was carried out in this group.

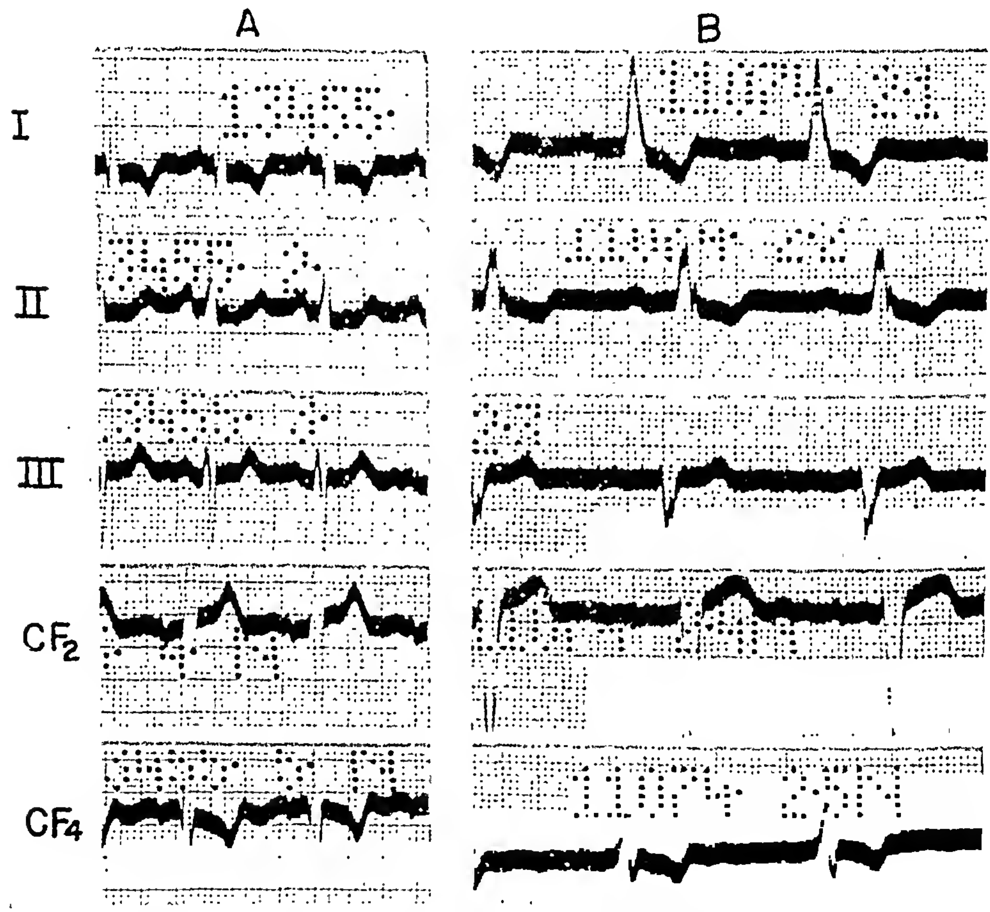


Fig. 5.—*A*. Second type of left ventricular preponderance. The U wave is inverted in Leads I and CF_4 and the T waves in these leads are also inverted. Note the U wave in Lead III is closely followed by the P wave and gives the false impression of a bizarre P wave (cf. Fig. 2). *B*. Intraventricular block of the common type. The U wave is inverted in Leads I and CF_4, and the T waves in these leads are inverted also.

Of the 135 cases of the common type of intraventricular block analyzed 17 had abnormal U waves; these findings are summarized in Table III. U-wave abnormalities occur most frequently in Leads I and CF_4 (Table VII). Thus a synthetic pattern in this type of intraventricular block would consist of inverted U waves in Leads I and CF_4 with normal upright or isoelectric U waves in Leads II and III and normal upright U waves in CF_2 (Fig. 5-*B*); this pattern is similar to that found in the second type of left ventricular preponderance.

TABLE II

U-WAVE CONFIGURATION IN LEFT VENTRICULAR PREPONDERANCE OF THE MIXED TYPE IN THE 11 CASES SHOWING U-WAVE ABNORMALITIES

LEAD	U INVERTED	U UPRIGHT AND NORMAL	U UPRIGHT AND ABNORMAL	U DIPHASIC		U ISO-ELECTRIC
				1ST PHASE UP	1ST PHASE DOWN	
I	8	0	0	0	0	3
II	1	2	0	0	0	8
III	0	4	0	0	1	6
CF_2	1	7	1	1	1	0
CF_4	5	5	0	0	1	0

TABLE III

U-WAVE CONFIGURATION IN COMMON TYPE OF INTRAVENTRICULAR BLOCK IN THE 17 CASES SHOWING U-WAVE ABNORMALITIES

LEAD	U INVERTED	U NORMAL AND UPRIGHT	U ISOELECTRIC	TOTAL CASES
I	13	0	4	17
II	1	5	11	17
III	0	7	10	17
CF_2	4	10	1	15*
CF_4	7	3	1	11†

*2 records had only 3 leads.
†6 records had no CF_4.

TABLE IV

U-WAVE CONFIGURATION IN ANTERIOR WALL INFARCTION PATTERN IN THE 18 CASES SHOWING U-WAVE ABNORMALITIES

LEAD	U INVERTED	U UPRIGHT AND NORMAL	U UPRIGHT AND ABNORMAL	U DIPHASIC		U ISO-ELECTRIC
				1ST PHASE UP	1ST PHASE DOWN	
I	6	0	0	0	0	12
II	1	4	0	0	0	13
III	0	10	0	0	0	8
CF_2	11	3	0	1	0	3
CF_4	16	0	0	1	0	1

TABLE V

U-WAVE CONFIGURATION IN POSTERIOR WALL INFARCTION PATTERN IN THE 11 CASES WITH U-WAVE ABNORMALITIES

LEAD	U INVERTED	U UPRIGHT AND NORMAL	U UPRIGHT AND ABNORMAL	U DIPHASIC		U ISO-ELECTRIC
				1ST PHASE UP	1ST PHASE DOWN	
I	3	2	0	0	0	6
II	1	2	0	0	0	8
III	2	2	0	0	0	7
CF_2	3	5	1	0	0	2
CF_4	9	1*	0	0	0	0

*One record had no CF_4.

THE U-WAVE PATTERNS IN MYOCARDIAL INFARCTION PATTERNS

Three hundred fifty cases with myocardial infarction patterns were analyzed, and in 39 abnormal U waves were seen. The latter included 18 of the anterior wall pattern, 11 of the posterior wall pattern, and 10 of the atypical patterns. Tables IV, V, and VI summarize the U-wave findings in these 39 cases.

In the anterior wall infarction series U-wave inversion was seen most frequently in CF_4 and next in frequency in CF_2 (Table VII). Thus, the idealized pattern of the U wave in anterior wall infarction consists of inversion of U in the chest leads, isoelectric or inverted U in Lead I, isoelectric U in Lead II, and isoelectric or upright U in Lead III (Fig. 6-*A*). Furthermore, analysis of 17 cases having serial curves showed that the U-wave abnormalities occurred only during the T stage and not the S-T stage.

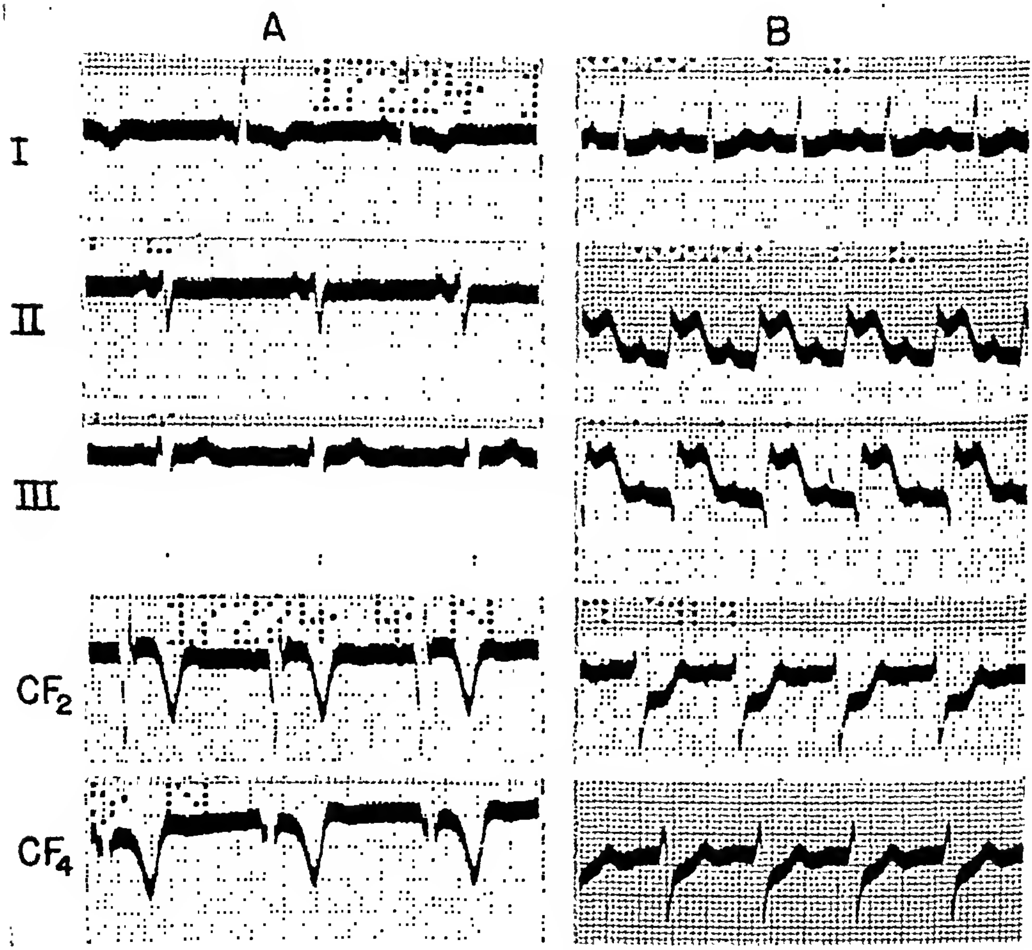


Fig. 6.—*A*. Anterior wall infarction in the healing (T) stage. The U wave in Leads I, CF_2 and CF_4 is inverted, and the U and T waves are concordant in direction in all leads. *B*. Very recent posterior wall infarction in the S-T stage (monophasic curve). The U waves in Leads CF_2 and CF_4 are inverted, and the S-T segments are markedly depressed. There is discordancy in direction between the U wave and T in these leads.

In the posterior wall infarction pattern Lead CF_4 again showed the greatest frequency of abnormal U waves (consisting of inversion) although scattered instances of abnormal U waves in the other leads were seen (Tables V and VII) (Fig. 6-*B*). Unlike the anterior wall infarcts, the U-wave abnormalities in posterior wall infarction were seen more commonly in the S-T stage.

TABLE VI

U-WAVE CONFIGURATION IN ATYPICAL MYOCARDIAL INFARCTION PATTERNS IN THE 10 CASES WITH U-WAVE ABNORMALITIES

LEAD	U INVERTED	U UPRIGHT AND NORMAL	U UPRIGHT AND ABNORMAL	U DIPHASIC		U ISO-ELECTRIC
				1ST PHASE UP	1ST PHASE DOWN	
I	2	1	0	1	0	6
II	1	2	0	0	0	7
III	4	5	0	0	0	1
CF_2	3	6	0	1	0	0
CF_4	8	0	0	2	0	0

TABLE VII

LEAD DISTRIBUTION OF ABNORMAL U WAVES IN THOSE INDIVIDUAL CASES SHOWING U-WAVE ABNORMALITIES

DIAGNOSIS	LEAD I	LEAD CF_4	LEADS I AND CF_4	LEAD CF_2	LEADS CF_2 AND CF_4	LEADS I, CF_2 AND CF_4	OTHER LEAD COMBINATIONS
Left Ventricular Preponderance, Mixed Type	4	0	2	0	3	1	1
Left Ventricular Preponderance, 2nd Type	9	4	4	0	2	1	3
Left Ventricular Preponderance, 1st Type	1	0	0	1	0	1	0
Left Ventricular Preponderance, Concordant Type	1	0	0	0	0	0	0
Intraventricular Block, Common Type	8	2	2	1	1	1	2
Anterior Wall Infarction Pattern	0	6	0	1	6	4	1
Posterior Wall Infarction Pattern	0	4	1	1	2	1	2
Atypical Infarction Patterns	0	4	1	0	4	0	1

Unlike the anterior and posterior wall patterns, the U-wave abnormalities in atypical infarction patterns occurred at all stages of the electrocardiographic evolution.

THE U-WAVE PATTERNS IN OTHER CONDITIONS

No abnormal U waves were found in the series of records of diffuse pericarditis, acute glomerulonephritis, or pulmonary embolism examined.

CORRELATION OF THE DIRECTION OF THE ABNORMAL U WAVES WITH THE DIRECTION OF THE T WAVE AND THE DEVIATION OF THE S-T SEGMENT

An impression was gathered in the course of this study that inverted U waves were usually associated with inverted T waves. In fact, Ashman and Hull[6] state that inverted U waves are commonly associated with inverted T waves. This was checked in all the records with regard to the concordancy of deviation of the U and T waves.

In left ventricular preponderance (all types) there was generally a concordancy in direction of the abnormal U waves and in the direction of T. This was most prominent in Leads I and CF_4 (Table VIII). This correlation was even more striking in the common type of intraventricular block since no instance of discordancy in direction of T and U occurred.

TABLE VIII

CORRELATION OF THE DIRECTION OF THE ABNORMAL U WAVE WITH THE DIRECTION OF THE T WAVE IN LEFT VENTRICULAR PREPONDERANCE AND IN THE COMMON TYPE OF INTRAVENTRICULAR BLOCK IN THE CASES WITH ABNORMAL U WAVES*

DIAGNOSIS	LEAD I		LEAD CF_2		LEAD CF_4	
	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT
Left Ventricular Preponderance, Mixed Type	8	0	3†	1	5	1
Left Ventricular Preponderance, Second Type	17	0	3†	3	11	3
Left Ventricular Preponderance, First Type	1	1	0	1	0	2
Left Ventricular Preponderance, Concordant Type	1	0	0	0	0	0
Intraventricular Block, Common Type	13	0	4	0	7	0

*A diphasic U wave was considered equivalent to an inverted one.
†2 U waves were inverted; one, upright and abnormal.

TABLE IX

CORRELATION OF DIRECTION OF ABNORMAL U WAVE WITH DIRECTION OF S-T DEVIATION AND WITH DIRECTION OF T WAVE IN CASES SHOWING ABNORMAL U WAVES*

DIAGNOSIS	S-T SEGMENT						T WAVE					
	LEAD I		LEAD CF_2		LEAD CF_4		LEAD I		LEAD CF_2		LEAD CF_4	
	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT	CONCORDANT	DISCORDANT
Anterior Wall Pattern	4	2	1	11	5	12	5	1	12	0	15	2
Posterior Wall Pattern	2	1	3	1	9	0	2	1	1	3	3	6
Atypical Patterns	1	2	0	4	5	5	2	1	3	1	7	3

*A diphasic U or T wave was considered equivalent to an inverted U or T.

In cases of anterior wall infarction pattern this concordancy was less striking but still fairly definite. However, in the posterior wall infarction pattern there was a definite discordancy in direction in T and U, especially in the chest leads. Accordingly, an analysis was made to ascertain a possible relationship between the direction of U and the deviation of the S-T segment.

In cases of anterior wall infarction there was a definite discordancy in the chest leads between the U and the deviation of the S-T segment (Table IX). A similar, although less definite, discordancy occurred in the series of atypical infarction patterns. On the other hand, in the posterior wall infarction patterns there was a definite concordancy in the direction of the U wave and the deviation of the S-T segment; this was most striking in Lead CF_4 (Table IX).

THE EVOLUTION OF THE U WAVE

An attempt was made to correlate the evolution of the abnormal U waves with that of the S-T-T segment in all cases having serial tracings.

In 15 of the 17 cases of left ventricular preponderance with serial tracings the abnormal U waves persisted. In the other 2 the abnormal U waves became normal even though the T waves remained inverted.

The U waves changed in appearance in 4 of the 8 cases of the common type of intraventricular block which had serial curves. In 2 of these the inverted U waves became isoelectric although the T waves remained inverted. The changes in the U wave in the other 2 cases were also independent of the T wave change.

In 6 of the 17 cases of anterior wall infarction pattern with serial tracings the abnormal U wave became normal. In 5 of these the change occurred in Lead CF_2, and, in all but one, the T wave in the lead remained inverted. It is significant that no abnormal U waves were seen when the S-T deviation was marked and the T wave small, even when the S-T deviation persisted for 10 months after the clinical attack.

In 5 of the 8 cases of posterior wall infarction pattern with serial records the abnormal U wave became normal. In contrast to the anterior wall infarction pattern U-wave abnormalities were noted in the posterior wall pattern during the period when the T was small and the S-T markedly deviated.

In 7 of the 9 cases of atypical infarction patterns with serial tracings the abnormal U waves changed to normal. These alterations in the U waves occurred without regard to the S-T or T changes.

It appears, therefore, that the initial appearance of the abnormal U wave is related to inversion of the T wave and to the depression of S-T in all the patterns studied except in myocardial infarction. In the latter, U inversion appears related to T inversion in the anterior pattern and to S-T depression in the posterior pattern. The U-wave evolution is sometimes independent of the S-T-T segment evolution.

Rapid appearance and disappearance of abnormal U waves were observed in 2 cases of transitory coronary insufficiency (with angina pectoris). A similar transitory occurrence of inverted U waves can be seen in 4 of the cases recently illustrated by Twiss and Sokolow.[6] In none of these 6 cases were records taken with sufficient frequency to determine whether or not the U-wave abnormality paralleled the abnormalities in the S-T-T segment. These cases do illustrate, however, how rapidly an abnormal U wave may appear and disappear.

DISCUSSION

Our results indicate that there is a tendency for the occurrence of a definite U-wave pattern in the abnormal electrocardiogram, although in most the U wave is normal in appearance in all the leads. When U-wave abnormalities occur in left heart strain and in intraventricular block of the common type the pattern consists, usually, of inverted U waves in Leads I and CF_4 with isoelectric or normal, upright U waves in the other leads. In anterior wall

infarction abnormal (inverted and diphasic) U waves occur most frequently in both Leads CF_2 and CF_4 with an occasional similar abnormality in Lead I, while in posterior wall infarction inverted U waves occur most commonly in CF_4 alone, although there are other scattered abnormalities. In all the patterns studied there was a general concordancy of the direction of the abnormal U wave with the direction of the T wave except in the early stages of posterior wall infarction pattern where there was a marked discordancy. In posterior wall infarction a definite concordancy existed with the deviation of the S-T segment as in other abnormal states expect in anterior wall infarction.

It must be re-emphasized that in these conditions many more instances of normal than of abnormal U waves were found.

The relationship of the abnormal U wave in posterior wall infarction to the deviation of the S-T segment rather than to the direction of the T wave is further supported by the rarity of abnormal U waves in Lead III in this pattern, in which lead, classically, the S-T segment is elevated and the T wave inverted. If abnormality of the U wave were related to the T-wave inversion, numerous instances of inverted U waves should be present in Lead III. On the other hand, in anterior wall infarction in which the S-T segment is depressed and the T wave is upright in Lead III abnormal U waves are not found, because, in this pattern, the U-wave abnormality appears to depend upon the direction of the T wave.

No abnormal U waves were found in the absence of other abnormalities in the electrocardiogram studied. Therefore, at the present, there is not much diagnostic value in the recognition of U-wave patterns; however, a description of the latter in abnormal records, such as has been attempted in this report, might aid in explaining the nature of the fundamental processes leading to the pattern of the U wave. Furthermore this knowledge will help to identify instances of distortion of the T and P waves and lead to a better definition of the isoelectric level.

SUMMARY

1. In 1000 cases with diagnostic electrocardiographic patterns, 94 cases of abnormal U waves were found. The U-wave patterns in these 94 cases showed the following chief characteristics:

(a) In left heart strain and in intraventricular block of the common type the U-wave abnormalities consist of inverted U waves in Leads II and CF_4.

(b) In anterior wall infarction abnormal (inverted or diphasic) U waves are found in Leads CF_2 and CF_4 with occasional similar abnormality in Lead I.

(c) In posterior wall infarction inverted U waves are found most commonly in Lead CF_4.

(d) No abnormal U waves were found in right heart strain or in intraventricular block of the indeterminate S type.

2. A hitherto undescribed U-wave abnormality, a negative bowing of the T-P segment in Lead I, is discussed.

3. Instances of distortion of the T and P waves by large upright U waves

and the confusion caused by the presence of prominent U waves in records of auricular fibrillation are pointed out.

4. Evidence is presented relating the presence of abnormal U waves to abnormalities of the S-T-T segment. In left heart strain and in the common type of intraventricular block, there is usually concordancy in the direction of the T wave, S-T deviation and U wave direction. In posterior wall infarction there is concordancy of the deviation of the S-T segment and the direction of the U wave but discordancy between T and U. In anterior wall infarction the reverse is true.

5. In serial records the abnormal U wave may undergo an evolution independent of the changes in the S-T-T segment.

We wish to express our grateful appreciation to Doctors L. N. Katz and R. Langendorf for their helpful criticisms.

REFERENCES

1. Papp, C.: U, the Sixth Wave of the Electrocardiogram, Brit. Heart J. 2: 9, 1940.
2. Nahum, L. H., and Hoff, H. E.: The Interpretation of the U Wave of the Electrocardiogram, Am. Heart J. 17: 585, 1940.
3. Katz, L. N., Goldman, A. M., Langendorf, R., Kaplan, L. G., and Killian, S. T.: The Diagnostic Value of the Electrocardiogram Based on an Analysis of 149 Autopsied Cases, Am. Heart J. 24: 627, 1942.
4. Kaplan, L. G., and Katz, L. N.: The Characteristic Electrocardiogram in Left Ventricular Strain With and Without Axis Deviation, Am. J. Med. Sci. 201: 676, 1941.
5. Katz, L. N.: Electrocardiography, Lea & Febiger, Philadelphia, 1941.
6. Ashman, R., and Hull, E.: Essentials of Electrocardiography, ed. 2, Macmillan Company, p. 166, 1941.
7. Twiss, A., and Sokolow, M.: Angina Pectoris, Am. Heart J. 23: 498, 1942.

INACTIVATION OF VACCINIA VIRUS BY MILD ANTISEPTICS*†

WOLCOTT B. DUNHAM, M.D., AND WARD J. MACNEAL, M.D., NEW YORK CITY

LITTLE is known concerning the inactivating effect of antiseptics on the viruses. The present study was undertaken as one of a series to elucidate this subject. It seemed particularly desirable to investigate the action of antiseptics on the vaccinia virus, as this virus has been the most studied in other respects.

A number of reports have appeared on the effect of a wide variety of chemical and physical agents on the vaccinia virus. A partial summary of this literature is shown in outline form in Table I. It will be noted that this list contains few bacterial antiseptics and that in most of the investigations, the virus was exposed to the agent for an hour or more. In the tests to be described,

*From the Department of Bacteriology, New York Post-Graduate Medical School and Hospital, Columbia University.

†Aided by the Virus Research Fund of the Lambert Pharmacal Company, St. Louis, Mo.
Received for publication, Sept. 19, 1942.

TABLE I

RESULTS OF PREVIOUS INVESTIGATIONS ON THE INACTIVATION OF THE VACCINIA VIRUS

AGENT	CONCENTRATION	TIME	REMARKS	RESULTS[a]	REFERENCES
Phenol	5%	1 hr.	Diluted Calf Lymph	(+)	2
Phenol	1%	1 hr.	Diluted Calf Lymph	–	2
Mercuric Bichloride	1:5000	1 hr.	Diluted Calf Lymph	+	2
Mercuric Bichloride	1:10,000	1 hr.	Diluted Calf Lymph	–	2
Ethyl Alcohol	50%	1 hr.	Diluted Calf Lymph	+	2
Ethyl Alcohol	20%	24 hr.	Diluted Calf Lymph	–	2
Methyl Alcohol	50%	1 hr.	Diluted Calf Lymph	+	2
Methyl Alcohol	20%	1 hr.	Diluted Calf Lymph	(+)	2
Methyl Alcohol	10%	24 hr.	Diluted Calf Lymph	–	2
Potassium Permanganate	1:100,000	1 hr.	Diluted Calf Lymph	+	2
Hydrogen Peroxide	1:10	1 hr.	Diluted Calf Lymph	+	2
Hydrogen Peroxide	1:50	1 hr.	Diluted Calf Lymph	–	2
Sodium Salicylate	1:10	1 hr.	Diluted Calf Lymph	+	2
Sodium Salicylate	1:50	1 hr.	Diluted Calf Lymph	–	2
Potassium Salicylate	0.25 M	4 hr.	Tissue Extract	(+)	3
Potassium Salicylate	0.4 M	1 hr.	Tissue Extract	+	3
Ether	50%	24 hr.	Diluted Calf Lymph	(+)	2
Ether.	20%	24 hr.	Diluted Calf Lymph	–	2
Acetone	50%	1 hr.	Diluted Calf Lymph	+	2
Acetone	10%	24 hr.	Diluted Calf Lymph	–	2
Formalin	0.25%	3 days	Washed Elementary Bodies	+	4
Chlorine	1:1,000,000	10 min.	Mandler Filtrate	–	5
Chlorine	1:500,000	10 min.	Mandler Filtrate	+	5
Hydrochloric Acid	N/1000	1 hr.	Diluted Calf Lymph	+	2
Sodium Hydroxide	N/500	1 hr.	Diluted Calf Lymph	+	2
Sodium Hydroxide	N/1000	24 hr.	Diluted Calf Lymph	–	2
Trypsin	0.5 mg. N per c.c.	3 wk.	Testicular Emulsion	–	6
Trypsin	0.5 mg. N per c.c.	1 wk.	Washed Elementary Bodies	(+)	6
Chymotrypsin	0.5 mg. N per c.c.	3 wk.	Washed Elementary Bodies	–	6
Saponin	0.2%	----	Chorio-allantoic Emulsion	(+)	7
Oil of Cloves	1:500	1 hr.	Diluted Calf Lymph	+	2
Drying	----	Years	Calf Lymph	–	8
Heat	55° C.	30 min.	Diluted Calf Lymph	+	2
Heat	50° C.	30 min.	Diluted Calf Lymph	–	2
Heat	100° C.	10 min.	Dry Virus	–	8
Cold	-8° C.	Years	Glycerinated Lymph	–	8
Pressure	400 Atmospheres	----	Glycerinated Lymph	–	8
Radium Radiation	----	----	------------	–	8
X-Ray	1.5 A	----	Testicular Emulsion	(+)	9
Light	Yellow, Green, Blue	----	Moist Virus	–	8
Light	Ultraviolet, Red	----	Moist Virus	+	8

[a] + Inactivation.
(+) Partial Inactivation.
– No Apparent Inactivation.

the vaccinia virus was exposed to the antiseptics for a period of only three minutes.

In a recent paper we[1] showed by means of chorio-allantoic inoculations that this virus is inactivated in 30 seconds by liquor antisepticus. The technic employed was rather time-consuming and it was not easy to make nearly simultaneous inoculations into a number of eggs. These difficulties were practically eliminated in the present study by injecting the mixtures of antiseptics and virus directly into the extra-embryonic fluids of the chick embryos. Two

advantages of this method are that a lot of 5 prepared eggs can be inoculated in half a minute and that the results tend to be remarkably uniform, apparently because the injurious effect of the needle puncture is slight. A disadvantage of this injection method is that localized pock lesions are seldom produced and hence one must, from time to time, test the virus by the chorio-allantoic method to be certain of its identity. Following inoculation into the extra-embryonic fluids, a generalized dissemination of the virus takes place and if the amount and activity of the inoculum are adequate, the embryo dies in two to four days. Death of the embryo is easily recognized by candling the eggs.

The virus suspension was prepared from incubated eggs which had died following injection of vaccinia virus CAEB, originally obtained from Doctor Joseph E. Smadel of the Rockefeller Institute. As a rule, the washed enveloping membrane of the yolk was used, but sometimes the tissue substance of the embryo itself and also the chorio-allantoic membrane were utilized. These tissues and membranes were ground with sand or glass granules and then mixed with Tyrode's solution, 1.5 c.c. for each yolk sac or chorio-allantois and 2 c.c. for each embryo body. The resulting suspension was centrifuged at 1,100 revolutions per minute for ten minutes. A pipette was then passed through the layer of yolk fat on top and the subjacent watery supernatant portion was withdrawn. This virus suspension was kept immersed in ice water until used. It was freshly prepared for each experiment and the virus strength was ascertained by titration of serial dilutions in each instance.

The eggs employed in the tests were incubated six to eight days, usually, seven days. Before use they were candled and only those with vigorous embryos were employed. These were marked to indicate the location of the embryo and the border of the air sac. The blunt end of the shell was treated with iodine and then alcohol and by means of a 2 millimeter dental bur, a hole was drilled through the shell without piercing the shell membrane. Several eggs thus prepared were placed in a holder with the embryo uppermost until the inoculations had been completed.

The test solutions were kept at room temperature before use. The virus suspension was at a temperature of about plus 4° C. In all instances nine parts of the test solution and one part of the virus suspension were vigorously mixed and allowed to stand for three minutes. At the end of this period the mixture was injected into the eggs directly or sometimes after dilution with sterile distilled water. The decision in regard to the dilution depended on the results of the toxicity tests previously reported by us[10] in which eggs were injected with antiseptics alone. Some mixtures were injected directly, others diluted tenfold and others a hundred-fold.

Just before the end of the three-minute exposure period, the shell openings of the prepared eggs were wiped with alcohol. The test mixture was then drawn into a tuberculin syringe fitted with a 2 inch, 20 gauge needle. The needle was passed through the hole in the egg shell and directed slightly upwards so that its point was about 12 millimeters below the mark on the shell indicating the location of the embryo, and 0.05 c.c. of the material was in-

jected. Examination of eggs inoculated in a similar manner with insoluble material has shown that the inoculum enters the allantoic cavity, or, occasionally, the amniotic cavity. After the injection, the needle was withdrawn without sidewise motion and the other eggs of the series were inoculated in rapid succession. Leakage through the puncture did not occur. The holes in the shells were then sealed with liquid adhesive and the eggs were returned to the incubator.

For each experiment the concentration of virus in the vaccinia suspension was determined by making ten-fold dilutions in series with distilled water and injecting each dilution into eggs. Ordinarily this series of dilutions extended from 10^{-1} to 10^{-8}. The titer of the original suspension was taken to be the highest dilution which killed half the embryos inoculated. The number of minimal lethal doses (M.L.D.) in 0.05 c.c. of the original suspension was taken to be the reciprocal of the titer. Thus where half the eggs were killed by inoculation of 0.05 c.c. of the 10^{-5} dilution, the titer of the original suspension was 10^{-5} and there were 100,000 M.L.D. in 0.05 c.c. of this original suspension. In no instances were tests of antiseptics considered significant unless the eggs received at least 10 M.L.D. of the virus. Beyond this minimum, the actual con-

TABLE II

RESULTS OF INOCULATIONS OF EGGS WITH MIXTURES OF VACCINIA VIRUS AND ANTISEPTICS. REACTION PERIOD 3 MINUTES. FIVE EGGS IN EACH TEST

AGENT	CONCENTRATION	SUBSEQUENT DILUTION OF MIXTURE	MORTALITY IN 6 DAYS
Phenol	5%	100	4/15
Phenol	3%	100	6/14
Phenol	1%	10	15/15
Tincture of Iodine, U.S.P. XI	1%	100	1/15
Tincture of Iodine, U.S.P. XI	0.1%	10	15/15
Mercuric Bichloride	1:1000	100	4/14
Mercuric Bichloride	1:10,000	10	14/14
Mild Silver Protein	20%	100	14/15
Copper Sulfate	1%	100	10/14
Potassium Permanganate	1:1000	10	15/15
Propylene Glycol	70%	----	2/15
Propylene Glycol	60%	----	13/15
Propylene Glycol	50%	----	15/15
Ethyl Alcohol	25%	----	15/15
Boric Acid	4%	10	14/15
Boric Acid in 25% Alcohol	2.5%	----	15/15
Liquor Antisepticus, N.F. VI	100%	----	4/14
Liquor Antisepticus, N.F. VI	80%	----	14/15
Liquor Antisepticus, N.F. VI Revised[a]	100%	----	7/15
Listerine	100%	----	2/15
Listerine	80%	----	14/15
Metaphen	1:500	10	13/15
Tincture of Metaphen (1:200)	25%	10	7/15
Potassium Mercuric Iodide	1:5000	----	15/15
Merthiolate	1:1000	100	15/15
Tincture of Merthiolate (1:1000)	25%	10	15/15
Lysol	2%	10	3/15
Lysol	0.5%	----	10/15
Lysol	0.1%	----	15/15
Mercurochrome	2%	10	14/15
Amphyl	1%	----	3/15
Amphyl	0.5%	10	14/15

[a]Prepared according to the officially revised formula, effective July 1, 1940.[11]

centration of virus in the suspension was found to be without effect on the outcome of the tests.

After inoculation the eggs were candled daily for six days. When an embryo appeared to be dead, as evidenced by loss of vascular markings, cloudiness of the embryonic region and increased passive mobility of the embryo, the egg was opened and examined. In the rare instances where the embryo was found malformed or had obviously died from hemorrhage, these facts were recorded and the egg was excluded from consideration in the determination of experimental results. Survival of the embryo for six days was accepted as evidence that the material injected contained less than one lethal dose of the active virus.

Each test was repeated at least three times, usually on different days with a newly prepared virus suspension in each instance. The results are summarized in Table II.

DISCUSSION

A certain amount of irregularity should be expected in biological experiments of this kind and we have not regarded the death of one embryo in a lot of five as of great significance. Occasionally an egg died as the result of obviously recognizable trauma and could therefore be excluded from consideration. Doubtless, an occasional death from unrecognized trauma also occurred. Furthermore, the virus suspension was not of entirely uniform composition. Although only the supernatant suspension, after centrifuging, was used, nevertheless this might well contain clumps and scattered particles of tissue elements which would furnish protection for contained virus elements and which might be irregularly distributed in the final suspension. Because of the very brief period of exposure for inactivation, three minutes, some irregularity due to this factor might well be anticipated. However, these irregularities have been so insignificant that they did not obscure the results.

As shown in Table II, 3 per cent phenol was moderately effective, while a 1 per cent solution failed to inactivate the virus. Tincture of iodine was effective when diluted to 1 per cent strength. This solution contained iodine in a concentration of 0.7 mg. per c.c. When the official tincture was diluted to 0.1 per cent strength, it was without effect. Bichloride of mercury was effective in a concentration of 1:1000, though a 1:10,000 solution failed to protect the embryos. When mixtures prepared from mild silver protein and virus were injected into eggs, 14 out of 15 of the embryos died. Copper sulfate, 1 per cent, showed some virucidal action. Potassium permanganate, 1:1000, failed to inactivate the virus under the conditions of the tests. A high degree of protection was afforded by a 70 per cent solution of propylene glycol in water, while a 50 per cent solution was without demonstrable effect. The concentration of propylene glycol in these mixtures was 63 per cent and 45 per cent respectively by volume, or 66 per cent and 47 per cent by weight. Full strength liquor antisepticus was effective, though in tests conducted with an 80 per cent solution, all but 1 of the eggs died. It was shown that the action of liquor antisepticus was not due to the 25 per cent alcohol or to the boric acid alone, as these agents when tested separately did not inactivate the virus.

A combination of the two in the proportion in which they are present in liquor antisepticus also failed to protect the embryos. Listerine, full strength, inactivated the virus, while an 80 per cent solution gave results similar to the same dilution of liquor antisepticus, N. F. VI. Commercial tincture of metaphen 1:200, diluted to one-quarter strength, inactivated the virus to such an extent that more than half the eggs survived. On the other hand, after inoculation with mixtures prepared from virus suspension and an aqueous solution of metaphen, 1:500, 13 of the 15 eggs died. Potassium mercuric iodide, 1:5000; merthiolate, 1:1000; and the commercial tincture of merthiolate, 1:1000, diluted to one-quarter strength, were without demonstrable effect. One egg survived out of 15 injected with mixtures containing 2 per cent mercurochrome and virus. Lysol, 2 per cent, inactivated the vaccinia virus in the 3-minute period. A 0.5 per cent solution gave some protection to the embryos, while all the eggs died which received mixtures containing 0.1 per cent lysol. A 1 per cent solution of amphyl inactivated the virus, whereas a 0.5 per cent solution was without appreciable effect.

The virus suspensions employed in the tests contained a considerable amount of protein, from 1 to 2 Gm. per 100 c.c. The mixtures of antiseptic and virus, therefore, contained 0.1 to 0.2 Gm. per 100 c.c. The action of certain of the antiseptics would undoubtedly be modified by the presence of this nonvirus protein. In nature, the viruses are associated with variable amounts of nonvirus protein, a fact which makes it desirable to test the action of an agent in the presence of both large and small amounts of protein. The protein content of the virus suspensions employed in the tests here described is probably comparable to that of most of the suspensions used in the tests reported by others and summarized in Table I, as the virus for almost all of those studies was obtained by emulsifying infected tissue or by collecting the lymph from vaccinia vesicles.

In a companion investigation,[12] we determined the action of antiseptics on the influenza virus by a similar technic, employing as the virus suspension extra-embryonic fluid from eggs which had died following inoculation with this virus. The protein content of this suspension was about 4.6 mg. per 100 c.c. The difference in concentration of nonvirus protein in the two sets of tests must be borne in mind if any attempt is made to derive from these results a comparison of the resistance of the influenza and vaccinia viruses to antiseptics.

SUMMARY

A number of antiseptics were tested for their effect on the vaccinia virus during a brief period of exposure. This was accomplished by preparing mixtures of the antiseptics and virus, allowing them to remain in contact for 3 minutes, diluting the mixture to a point where it would not be toxic for chick embryos and then injecting the material into embryonated eggs. Survival of the embryos indicated inactivation of the virus.

The following agents were effective in inactivating the vaccine virus in 3 minutes: phenol, 3 per cent; tincture of iodine, 1 per cent; bichloride of

mercury, 1:1000; propylene glycol, 70 per cent; liquor antisepticus, N. F. VI, undiluted; listerine, undiluted; tincture of metaphen, 1:200, diluted to one-quarter strength; lysol, 2 per cent; and amphyl, 1 per cent.

REFERENCES

1. Dunham, Wolcott B., and MacNeal, Ward J.: Culture on the Chick Chorio-allantois as a Test of Inactivation of Vaccinia Virus, J. Bact. 44: 413, 1942.
2. Gordon, M. H.: Studies of the Viruses of Vaccinia and Variola, Privy Council, Medical Research Council, Special Report Series, No. 98, London, 1925.
3. Cooke, Barbara, and Best, Rupert J.: Inactivating Effect of Salicylate on Suspensions of Some Animal Viruses. (Herpes, Infectious Myxoma, Shope Fibroma, Vaccinia and Poliomyelitis), Australian J. Exper. Biol. & M. Sc. 19: 93, 1941.
4. Weil, A. J., and Gall, L. S.: Studies on the Immunization of Rabbits With Formalized Vaccine Virus, J. Immunol. 38: 1, 1940.
5. MacDonald, Hugh: Chlorine Death Point of Cowpox Virus, Illinois M. J. 77: 356, 1940.
6. Merrill, Malcolm H.: Effect of Purified Enzymes on Viruses and Gram-negative Bacteria, J. Exper. Med. 64: 19, 1936.
7. Burnet, F. M., and Lush, D.: The Action of Surface-active Agents on Viruses, Australian J. Exper. Biol. & M. Sc. 18: 141, 1940.
8. Paschen, E.: Pocken, Resistenzverhältnisse. Kolle, W., Kraus, R., and Uhlenhuth, P.: Handbuch der pathogenen Mikroorganismen, Ed. III, Band VIII: 870, Jena, Berlin, Wien, 1930, Gustav Fischer and Urban and Schwarzenberg.
9. Gowen, John W., and Lucas, Alfred M.: Reaction of Variola Vaccine Virus to Roentgen Rays, Science 90: 621, 1939.
10. Dunham, Wolcott B.: Toxicity of Antiseptics for the Chick Embryo, Proc. Soc. Exper. Biol. & Med. 50: 274, 1942.
11. Interim Revision Announcement, No. 2. National Formulary. Sixth Edition. J. Am. Pharm. 28: 1061, 1939.
12. Dunham, Wolcott B., and MacNeal, Ward J.: Inactivation of Influenza Virus by Mild Antiseptics, J. Bact. 43: 85, 1942.

THE COMPARATIVE EFFECTIVENESS OF ARSENICAL COMPOUNDS AND SULFONAMIDE DRUGS AGAINST BACTERIAL INFECTIONS*

EDWIN E. OSGOOD, M.D., PORTLAND, OREGON

WITH THE TECHNICAL ASSISTANCE OF JANE M. ARMENTROUT, M.A.; DOROTHY M. ELLIS, B.A.; INEZ E. BROWNLEE, B.A.; AND JULIA JOSKI, M.A.

IN PREVIOUS publications it has been shown, experimentally by the technique of marrow culture,[1] and clinically in staphylococcic bacteriemias[2] and cases of subacute bacterial endocarditis,[3] that neoarsphenamine is more effective than drugs of the sulfonamide group against the *Staphylococcus aureus* and most strains of the *Streptococcus viridans*. The present study was undertaken to determine in cultures of living human marrow cells the comparative effectiveness of neoarsphenamine, many other arsenicals, and the four sulfonamide drugs in most common use against the *Staphylococcus aureus*, the *Streptococcus viridans*, and many species of bacteria not previously reported on.

*From the Department of Medicine and the Division of Experimental Medicine, University of Oregon Medical School, Portland, Oregon.

Aided by grants from Mr. John C. Higgins, The Department of Clinical Research, Abbott Laboratories, and The General Research Council of the Oregon State System of Higher Education. The drugs used in the experiments were supplied by the Abbott Laboratories, the Lederle Laboratories, the Winthrop Chemical Company, and E. R. Squibb and Sons.

Received for publication, Sept. 21, 1942.

Clinically and in animal experimentation, control of such variables as the size of the initial inoculation, the number and strain of organisms present, the concentration of the drug in the blood and tissues during therapy, the day of disease on which therapy is started, the natural resistance of the host, and the presence or absence of antibodies is difficult, if not impossible, to attain. By the use of the method of human marrow culture[4] these variables are controlled and quantitative studies are possible. In addition, it is possible to study the toxicity of the drugs to living human cells.

METHOD

About 10 c.c. of marrow obtained by sternal puncture[5] or leukemic blood were introduced into 25 c.c. of citrated balanced salt solution in a 50 c.c. vaccine vial. After centrifugation the buffy coat was transferred to another vial containing balanced salt solution, and a total nucleated cell count was done and the culture diluted to a final volume of 50 to 100 c.c. containing 100,000,000 to 200,000,000 nucleated cells. The medium consisted of 65 per cent balanced salt solution similar in composition to cerebrospinal fluid and 35 per cent human cord serum or ascitic fluid. To this was added the desired inoculum of the bacteria to be studied and, after mixing, pour plates for colony counts were made on a medium suitable for the growth of the organism. These represent the initial colony counts at 0 time in the tables. These cultures were mixed again, and equal volumes were transferred to a series of six to fifteen 30 c.c. vaccine vials. All manipulations were made with syringe and needle through 70 per cent alcohol on vaccine caps. To each of these vials, except the control, the desired concentration of the drug to be studied was added, and a volume of saline equal to that in which the drugs were dissolved was added to the control vial. The vials were then transferred to an incubator at 37° C., and at intervals pour plates for colony counts and stained smears were made. This technique insures that the cultures were identical in every respect except for the single variable of the drug introduced.

RESULTS

A total of 211 such experiments on 118 different strains representing 11 different species of bacteria was performed. All of the 211 such experiments were analyzed in drawing the conclusions in this paper concerning the relative effectiveness of different drugs. Unless colony counts in the same experiment at the same time for different drugs or different concentrations of the same drug differed by a ratio of 10:1 they were not regarded as significantly different. In Tables I to V the results of 5 of the 211 experiments are tabulated.

Note from Table I that against this small inoculum of *Staphylococcus aureus* arsenic trioxide and the pentavalent arsenicals, acetarsone, aldarsone and carbarsone, are almost totally ineffective; that sulfathiazole in a concentration of 10 mg. per 100 c.c. is about equally effective with sulfadiazine at twice this concentration; and that all of the trivalent organic arsenicals used in this experiment are much more effective in the concentrations employed than are either sulfathiazole or sulfadiazine. In this particular experiment neoarsphenamine in a concentration of 0.5 mg. per 100 c.c. is about equal in effectiveness to 0.33

TABLE I

Experiment 614. *Staphylococcus areus*, Lewis

HOURS	0	20	24	44
Control	84	1,000,000,000		
Neoarsphenamine, 0.5 mg. per 100 c.c.	84	360	960	5,100
Arsphenamine, 0.33 mg. per 100 c.c.	84	200	340	2,300
Mapharsen, 0.33 mg. per 100 c.c.	84	880	820	260,000
Mapharsen, 0.1 mg. per 100 c.c.	84	800	1,900	61,000,000
Trisodarsen, 0.5 mg. per 100 c.c.	84	2,700,000	12,000,000	400,000,000
Arsenic trioxide, 0.15 mg. per 100 c.c.	84	800,000,000		
Acetarsone, 0.4 mg. per 100 c.c.	84	1,000,000,000		
Aldarsone, 0.6 mg. per 100 c.c.	84	1,000,000,000		
Carbarsone, 0.4 mg. per 100 c.c.	84	800,000,000		
Sulfathiazole, 10 mg. per 100 c.c.	84	6,900,000	43,000,000	720,000,000
Sulfadiazine, 20 mg. per 100 c.c.	84	7,000,000		

TABLE II

Experiment 713. *Staphylococcus aureus*, Schildon

HOURS	0	20	46	68
Control	151	900,000,000	2,220,000,000	
Neoarsphenamine, 0.5 mg. per 100 c.c.	151	20	12	3
Arsphenamine, 0.3 mg. per 100 c.c.	151	600	400	60
Sulfarsphenamine, 0.33 mg. per 100 c.c.	151	360	100	35
Trisodarsen, 0.5 mg. per 100 c.c.	151	1,200	28,000	3,500
Mapharsen, 0.2 mg. per 100 c.c.	151	20	8	0
Mapharsen, 0.05 mg. per 100 c.c.	151	19,200	160,000,000	8,000,000,000
Clorarsen, 0.5 mg. per 100 c.c.	151	13	2	0
Clorarsen, 0.05 mg. per 100 c.c.	151	60,000	40,000,000	2,400,000,000
Sulfapyridine, 10 mg. per 100 c.c.	151	18,000,000	9,000,000,000	
Sulfathiazole, 10 mg. per 100 c.c.	151	80	13	5
Sulfadiazine, 10 mg. per 100 c.c.	151	100,000,000	5,000,000,000	

mg. per 100 c.c. of arsphenamine and only slightly superior in effectiveness to 0.33 mg. per 100 c.c. of mapharsen. It is far superior to 0.1 mg. per 100 c.c. of mapharsen and to 0.5 mg. per 100 c.c. of trisodarsen.

Note by comparison of Tables I and II that the strain of *Staphylococcus aureus* used in the experiment in Table II is far more susceptible to the action of all drugs than the strain employed in Table I. A larger inoculum is sterilized by concentrations of the drugs which failed to sterilize the strain used in Table I.

Note in Table II that in this experiment 0.5 mg. per 100 c.c. of neoarsphenamine, 0.2 mg. per 100 c.c. of mapharsen, 0.5 mg. per 100 c.c. of clorarsen, and 10 mg. per 100 c.c. of sulfathiazole are about equally effective; that 0.3 mg. per 100 c.c. of arsphenamine and 0.33 mg. per 100 c.c. of sulfarsphenamine are highly and equally effective; that 0.5 mg. per 100 c.c. of trisodarsen is definitely not as effective; and that mapharsen in a concentration one-tenth that of neoarsphenamine, which is about the ratio of their clinical doses, is not nearly as effective as neoarsphenamine but is more effective than an equal concentration of clorarsen. Also note how much less effective sulfapyridine and sulfadiazine are than is sulfathiazole in equal concentration.

Note in Table III that this strain of *Streptococcus viridans* is highly susceptible to the action of each of the trivalent organic arsenicals investigated but that clorarsen and mapharsen in one-tenth the concentration of neoarsphenamine are not nearly as effective.

TABLE III

Experiment 723. *Streptococcus viridans*, Winberg

HOURS	0	20	49	68
Control	33	185,000	2,800,000	
Neoarsphenamine, 0.5 mg. per 100 c.c.	33	5	2	0
Arsphenamine, 0.3 mg. per 100 c.c.	33	5	1	0
Sulfarsphenamine, 0.33 mg. per 100 c.c.	33	90	1	0
Clorarsen, 0.5 mg. per 100 c.c.	33	0	2	0
Clorarsen, 0.4 mg. per 100 c.c.	33	1	3	0
Clorarsen, 0.05 mg. per 100 c.c.	33	150	260	26
Mapharsen, 0.3 mg. per 100 cc..	33	1	1	0
Mapharsen, 0.2 mg. per 100 c.c.	33	4	0	0
Mapharsen, 0.05 mg. per 100 c.c.	33	1,700	12,500	700
Trisodarsen, 0.5 mg. per 100 c.c.	33	60	2	0

TABLE IV

Experiment 717. *Streptococcus hemolyticus*, Schuerman

HOURS	0	22	46
Control	4,600	19,000,000*	
Neoarsphenamine, 0.5 mg. per 100 c.c.	4,600	0	0
Arsphenamine, 0.3 mg. per 100 c.c.	4,600	0	0
Sulfarsphenamine, 0.33 mg. per 100 c.c.	4,600	0	0
Trisodarsen, 0.5 mg. per 100 c.c.	4,600	130	0
Mapharsen, 0.2 mg. per 100 c.c.	4,600	0	0
Mapharsen, 0.05 mg. per 100 c.c.	4,600	600,000	10,000
Clorarsen, 0.5 mg. per 100 c.c.	4,600	0	0
Clorarsen, 0.05 mg. per 100 c.c.	4,600	1,900,000	10,000
Sulfanilamide, 10 mg. per 100 c.c.	4,600	110,000,000	
Sulfapyridine, 10 mg. per 100 c.c.	4,600	70,000,000	
Sulfathiazole, 10 mg. per 100 c.c.	4,600	1,800,000	16,000,000
Sulfadiazine, 10 mg. per 100 c.c.	4,600	100,000,000	

*Acid at 18 hours.

TABLE V

Experiment 719. *Streptococcus hemolyticus*, Schuerman

HOURS	0	4	24
Control	54,000	10,000,000	40,000,000
Neoarsphenamine, 0.5 mg. per 100 c.c.	54,000	7,000	0
Arsphenamine, 0.3 mg. per 100 c.c.	54,000	30,000	10
Sulfarsphenamine, 0.33 mg. per 100 c.c.	54,000	40,000	0
Mapharsen, 0.3 mg. per 100 c.c.	54,000	5,600	0
Mapharsen, 0.2 mg. per 100 c.c.	54,000	9,000	0
Mapharsen, 0.05 mg. per 100 c.c.	54,000	100,000	210,000
Clorarsen, 0.5 mg. per 100 c.c.	54,000	4,200	0
Clorarsen, 0.4 mg. per 100 c.c.	54,000	5,000	0
Clorarsen, 0.05 mg. per 100 c.c.	54,000	200,000	90,000

Note in Table IV that the *Streptococcus hemolyticus* (beta hemolytic streptococcus, Lancefield group A) is far more susceptible to the action of the trivalent organic arsenicals than is the staphylococcus, since a larger inoculum is sterilized by the same concentrations of the drugs which failed to sterilize small inoculums of the Staphylococcus aureus. It is again evident in this experiment that trisodarsen in equal concentration is not as effective as neoarsphenamine; that mapharsen in one-tenth the concentration is not nearly as effective as neoarsphenamine; that the trivalent organic arsenicals are all far superior to the sulfonamide drugs; and that sulfathiazole is much the most effective of the sulfonamide drugs.

Note from a comparison of Tables IV and V that it is necessary to increase the initial inoculum to demonstrate the differences in effectiveness of the most effective drugs and to decrease the initial inoculum to demonstrate the differences in effectiveness of the least effective drugs.

Note in Table V the great susceptibility of this strain of *Streptococcus hemolyticus* to the trivalent organic arsenicals and that 0.5 mg. per 100 c.c. of neoarsphenamine is about equal in effectiveness to 0.25 mg. of mapharsen and 0.4 mg. per 100 c.c. of clorarsen; that 0.3 mg. per 100 c.c. of arsphenamine and 0.33 mg. per 100 c.c. of sulfarsphenamine are almost as effective but that one-tenth the concentration of mapharsen and clorarsen are not nearly as effective.

COMMENT

Analyses of the 211 experiments on the 118 strains representing 11 different species of bacteria were made. The pentavalent organic arsenicals studied and arsenic trioxide were almost totally ineffective when compared with effective concentrations of neoarsphenamine, either in the ratios of their clinical doses or in concentrations which would give an equal arsenic content. The pentavalent arsenicals studied included acetarsone, carbarsone, aldarsone, para-sulfonamindophenyl arsonic acid, tryparsamide, sodium cacodylate, and solarson. Only solarson seemed to be of any value at all, and this was far less effective than neoarsphenamine.

All of the trivalent organic arsenicals tested were highly effective against certain species of bacteria in concentrations of equal arsenic concentration to the effective range for neoarsphenamine previously determined to be 75 to 200 gammas (micrograms) per 100 c.c.,[6] corresponding to 0.375 to 0.1 mg. per 100 c.c. of neoarsphenamine. The trivalent organic arsenicals tested were neoarsphenamine, arsphenamine, sulfarsphenamine, trisodarsen, mapharsen, clorarsen, bismarsen, and silver arsphenamine. The latter two offered no advantages for clinical use, since they were relatively ineffective in comparison to the other arsenicals; so they were not studied very extensively.

The different species of bacteria and in some instances even different strains of the same species differed markedly in their susceptibility to the action of the drugs. When an organism was susceptible to the action of one of these arsenicals the same strain was always highly susceptible to the action of the other trivalent organic arsenicals when compared in doses having an equal arsenic content. When the organism was resistant to the action of one arsenical it was resistant to the action of the others as well, although this was not extensively investigated. While there were individual strain differences in susceptibility with each of the organisms tested, only with the *Streptococcus viridans* was there a sufficient difference to justify grouping these strains into separate categories. As pointed out in previous publications,[6] some strains of *Streptococcus viridans* respond to neoarsphenamine and not at all to the sulfonamide drugs, and these strains have arbitrarily been called "Group A." Other strains are susceptible both to the action of neoarsphenamine and to the sulfonamide drugs, and these have been arbitrarily called "Group B." Other strains have been susceptible to the action of the sulfonamide drugs and not to neoarsphenamine, and these have arbitrarily been called "Group C."

In Table VI, the response of the bacteria tested to the trivalent organic arsenicals is summarized. Note that the *Staphylococcus aureus*, *Streptococcus viridans* (Groups A and B), *Streptococcus hemolyticus* (betahemolytic streptococcus, Lancefield group A), *Corynebacterium diphtheriae*, *Neisseria gonorrhoeae*, and *Hemophilus influenzae* are highly susceptible to the action of these drugs; whereas, the *Diplococcus pneumoniae*, *Escherichia coli*, *Eberthella typhosa*, *Salmonella schottmuelleri*, *Streptococcus viridans* (Group C), and *Streptococcus anhemolyticus* (gamma streptococcus) were almost totally unaffected by the same concentrations of these trivalent organic arsenicals. Most strains of the first six species of bacteria in Table VI were far more susceptible to the action of the trivalent arsenicals in the concentrations employed than they were to the usual concentrations of the sulfonamide drugs obtainable in the blood stream. *Neisseria gonorrhoeae*[7] and *Hemophilus influenzae*, however, were somewhat less susceptible to the action of the trivalent organic arsenicals than to sulfathiazole, sulfadiazine, and sulfapyridine, although they were more susceptible to the action of the trivalent arsenicals than to the action of sulfanilamide.

TABLE VI

SUMMARY OF THE RESPONSE OF BACTERIA TO TRIVALENT ORGANIC ARSENICALS

ARSENICALS EFFECTIVE	ARSENICALS INEFFECTIVE
Staphylococcus aureus	*Diplococcus pneumoniae*
Streptococcus viridans	*Escherichia coli*
Group A	*Eberthello typhosa*
Group B	*Salmonello schottmuelleri*
Streptococcus hemolyticus	*Streptococcus viridans*
Corynebacterium diphtheriae	Group C
Neisseria gonorrhoea	*Streptococcus anhemolyticus*
Hemophilus influenzae	

Against each of the organisms listed in Table VI, sulfathiazole was the most effective of the sulfonamide drugs and sulfanilamide was the least effective. All of these organisms, except the Group A *Streptococcus viridans*, showed some response to 10 mg. per 100 c.c. of sulfathiazole if small enough initial inoculums were used. Sulfapyridine was superior to sulfadiazine in equal concentration against most strains of all of the species in Table VI, except *Neisseria gonorrhoeae* and *Salmonella schottmuelleri* where sulfadiazine was as a rule superior to sulfapyridine.

While there were rather marked individual strain differences among organisms of the same species or group there was a very great variation in susceptibility of the different species of organisms to the trivalent organic arsenicals and to the sulfonamide drugs, as indicated by the maximum initial inoculums which could be sterilized.

Note from Table VII that the *Streptococcus hemolyticus* is the most susceptible organism to the action of the trivalent organic arsenicals and that the *Neisseria gonorrhoeae* is the most susceptible organism to the action of the sulfonamide drugs. For each group of drugs the organisms can be divided into five or six groups of markedly different susceptibilities.

Since six of the trivalent organic arsenicals tested were about equally effective in terms of equal arsenic content, it seemed desirable to calculate a thera-

TABLE VII

COMPARATIVE SUSCEPTIBILITY OF ORGANISMS TO NEOARSPHENAMINE AND SULFATHIAZOLE

NEOARSPHENAMINE, 0.5 MG. PER 100 C.C.		SULFATHIAZOLE, 5-10 MG. PER 100 C.C.	
ORGANISMS	MAXIMUM INOCULUM STERILIZED	ORGANISMS	MAXIMUM INOCULUM STERILIZED
Str. hemolyticus	800,000	*N. gonorrhoeae*	1,000,000
N. gonorrhoeae	20,000	*Str. hemolyticus*	6,000
Str. viridans (Groups A and B)	1,500-3,000	*Str. viridans* (Groups B and C)	1,500-3,000
H. influenzae	1,500-3,000	*Dip. pneumoniae*	1,500-3,000
C. diphtheriae	1,500-3,000	*H. influenzae*	1,500-3,000
Staph. aureus	750	*Staph. aureus*	100-300
Sal. schottmuelleri	Relatively ineffective	*C. diphtheriae*	100-300
E. typhosa	Relatively ineffective	*Sal. schottmuelleri*	100-300
E. coli	Relatively ineffective	*E. typhosa*	100-300
Str. anhemolyticus	Relatively ineffective	*E. coli*	100-300
Dip. pneumoniae	Relatively ineffective	*Str. anhemolyticus*	100
Str. viridans (Group C)	Relatively ineffective	*Str. viridans* (Group A)	Ineffective

TABLE VIII

COMPARATIVE EFFECTIVENESS OF THE TRIVALENT ORGANIC ARSENICALS

DRUG	PERCENTAGE OF ARSENIC	EQUALLY EFFECTIVE CONCENTRATION		MAXIMUM THERAPEUTIC DOSE		THERAPEUTIC INDEX	
		MG. PER 100 C.C.	GAMMAS, AS. PER 100 C.C.	GM. PER 60 KG.*	MG. PER 100 GM.		NEO = 100
Neoarsphenamine	20.0	0.5	100	0.9	1.5	3.0	100.0
Sulfarsphenamine	19.0	0.5	95	0.6	1.0	2.0	66.7
Arsphenamine	31.0	0.35	110	0.4	0.67	1.9	63.3
Trisodarsen	19.0	0.6	114	0.6	1.0	1.67	55.7
Mapharsen	29.0	0.25	75	0.06	0.1	0.4	13.3
Clorarsen	25.5	0.4	102	0.067	0.11	0.275	9.0

*From Moore, J. E., et al.: The Modern Treatment of Syphilis, p. 76. Charles C Thomas, Springfield, 1941.

†Mg. per 100 Gm. (dose) ÷ Mg. per 100 c.c. (concentration).

peutic index to determine which of them should be most promising for clinical use. It has been previously determined[6] that the effective range of neoarsphenamine in clinical use is 0.375 to 1.00 mg. per 100 c.c. of blood corresponding to 75 to 200 gammas (micrograms) of arsenic per 100 c.c. Therefore, neoarsphenamine in a concentration of 0.5 mg. per 100 c.c. corresponding to 100 gammas of arsenic per 100 c.c. was used as a reference standard, and all experiments in which this concentration of neoarsphenamine was compared with one or more of the other five arsenicals were analyzed. From these data, Table VIII was prepared. The first vertical column in Table VIII lists the commonly used names of the six trivalent organic arsenicals which offer most promise in the treatment of bacterial infections. The second column gives the percentage of arsenic in the drug as it is supplied in ampoules, which is not always the percentage as it is calculated from the structural formula. The third column gives the concentration in milligrams per 100 c.c. of these drugs as commercially supplied which, from analysis of all of the experiments in which the drugs were compared, appeared to be equivalent to 0.5 mg. per 100 c.c. of neoarsphenamine. The fourth column gives the gammas (micrograms) of arsenic per 100 c.c. which correspond to the amount of drug listed in the third column. From examination of this

column it is apparent that the drugs are approximately equally effective in concentrations of equal arsenic content, with the exception of mapharsen which is slightly more effective than the others in terms of equal arsenic content. The fifth column is the maximum therapeutic dose in grams per 60 kilograms as given by J. E. Moore,[8] with the exception of clorarsen for which the dose is that of the largest ampoule commonly administered. Column 6 is the milligrams per 100 grams of body weight calculated from column 5. Column 7 is a therapeutic index, which was calculated by dividing column 6 by column 3. Column 8 is the same index transposed to a basis of "neoarsphenamine = 100" by dividing all of the values by 3 in column 7 and multiplying by 100. From this column it is readily apparent that in the ratios of the maximum therapeutic doses, neoarsphenamine is by far the most effective drug, sulfarsphenamine and arsphenamine next, trisodarsen less effective, and mapharsen and clorarsen much less effective. Similar indexes were calculated, based on the maximum toxic concentraton for marrow cells, and the maximum tolerated dose in grams per kilogram from the same table,[6] but these indexes showed an even greater superiority of neoarsphenamine and inferiority of mapharsen and clorarsen, so are not included in this article.

It is fortunate that neoarsphenamine proves to be the trivalent organic arsenical of choice in the therapy of bacterial infections, since a method of administration[6] to obtain an effective blood level for 50 per cent of the time over a period of 60 days has been developed from a series of blood arsenic determinations. It has been shown to be effective, if followed exactly, in several patients with staphylococcic bacteriemia and subacute bacterial endocarditis. It maintains a more uniform blood level than with the massive intravenous drip technique[9] over a much longer period and yet less arsenic is administered per unit period of time. It does, of course, introduce much more arsenic than the standard clinical group method of treating syphilis but it does not at any time raise the blood level nearly as high as the blood level rises after a single intravenous injection of 0.6 Gm. of neoarsphenamine in a 60 kilogram person.

Comparative analysis in a similar way of the equally effective concentrations of the sulfonamide drugs indicated that 5.0 mg. per 100 c.c. of sulfathiazole is approximately equal in effectiveness to 10 mg. per 100 c.c. of sulfapyridine, 20 mg. per 100 c.c. of sulfadiazine, and 30 mg. per 100 c.c. of sulfanilamide. There were marked individual strain variations in this susceptibility. It is apparent from this that sulfathiazole is the drug of choice among the sulfonamide drugs for therapy of infections due to the organisms listed in Table VI, except when toxic reactions, danger of meningitis, or the presence of meningitis indicates the use of sulfadiazine or sulfapyridine. Sulfanilamide was so much less effective than the other drugs that there seems to be little reason for using it further in the therapy of infections with an organism listed in Table VI. A more detailed presentation of the experimental evidence and the conclusions in regard to each of these organisms listed in Table VI will appear elsewhere in separate papers for each organism. It has already appeared for *Neisseria gonorrhoeae*.[7]

SUMMARY

Analysis of 211 experiments similar to those in Tables I to V on 118 strains of 11 different species of bacteria by the marrow culture technique comparing the

effectiveness of arsenical compounds and the four commonly used sulfonamide drugs is presented. This analysis indicates that in marrow cultures arsenic trioxide and the seven pentavalent organic arsenicals tested are ineffective against all of the species of bacteria studied in therapeutically feasible concentrations. All of the trivalent organic arsenicals ordinarily used in the therapy of syphilis are effective in marrow cultures against the six species of bacteria listed in column 1 of Table VI in concentrations corresponding to about 100 gammas of arsenic per 100 c.c. These trivalent organic arsenical compounds were almost completely ineffective in this concentration against the five species of organisms in column 2 of Table VI. The trivalent organic arsenicals at this concentration were more effective against most strains of the *Staphylococcus aureus, Streptococcus hemolyticus, Streptococcus viridans,* and *Corynebacterium diphtheriae* than the most effective of the sulfonamide drugs in concentrations of 5.0 to 10.0 mg. per 100 c.c. The trivalent organic arsenicals when compared in these concentrations were not as effective as sulfathiazole or sulfadiazine against the *Neisseria gonorrhoeae* and *Hemophilus influenzae* but were more effective than sulfanilamide. When effective at all, the equally effective concentrations of the six more commonly used trivalent organic arsenicals are shown in Table VIII, with the corresponding arsenic concentrations, maximum tolerated therapeutic doses, and a therapeutic index, indicating their relative promise of effectiveness in clinical use against bacterial infections. There was no consistent correlation between the effective concentrations of the trivalent arsenicals and the sulfonamide drugs tested. The equally effective concentrations among the sulfonamide drugs tested are sulfathiazole, 5.0 mg. per 100 c.c.; sulfapyridine, 10 mg. per 100 c.c.; sulfadiazine, 20 mg. per 100 c.c.; and sulfanilamide, 30 mg. per 100 c.c.

The bacteria tested showed marked species and strain differences in susceptibility to the action of the chemotherapeutic agents, as indicated by the size of the inoculum which could be sterilized by a given concentration, as shown in Table VII. The *Streptococcus hemolyticus,* as shown in Table VII, was the most susceptible organism to the action of the trivalent organic arsenicals, and the *Neisseria gonorrhoeae* was most susceptible to the action of the sulfonamide compounds.

CONCLUSIONS

Neoarsphenamine in a concentration which can be maintained 50 per cent of the time for 60 days clinically, if the directions for administration previously published[6] are followed exactly, deserves further trial in the therapy of subacute bacterial endocarditis and bacteriemias, endocarditis, or other serious infections due to the *Staphylococcus aureus, Streptococcus hemolyticus, Hemophilus influenzae, Neisseria gonorrhoeae,* and, possibly, as a local application in diphtheria. Sulfarsphenamine and arsphenamine are somewhat less and mapharsen and clorarsen are much less effective than neoarsphenamine when compared in concentrations attainable clinically.

Sulfathiazole would appear to be the drug of choice among the sulfonamide drugs now available for clinical use, with sulfadiazine to be substituted should sulfathiazole not be tolerated, and sulfapyridine or sulfadiazine to be used if meningitis is present or threatened.

REFERENCES

1a. Osgood, E. E.: Marrow Cultures. A Symposium on the Blood, Madison, 1939, University of Wisconsin Press, pp. 219-241.
b. Osgood, E. E.: Culture of Human Marrow: Studies of the Relative Effectiveness of Neoarsphenamine, Mapharsen, Sulfanilamide, Sulfapyridine, Sulfathiazole, and Sulfamethylthiazole on Infections With Streptococcus Viridans (Alpha Hemolytic Streptococcus), Am. J. M. Sc. 200: 596, 1940.
c. Osgood, E. E., Joski, Julia, and Brownlee, Inez E.: The Superiority of Neoarsphenamine and Sulfathiazole in the Therapy of Staphylococcus Aureus Infections in Marrow Cultures, Surg., Gynec. & Obst. 71: 445, 1940.
2. Osgood, E. E.: The Chemotherapy of Staphylococcic Infections, J. Pediat. 17: 740, 1940.
LeCocq, E.: Use of Neosalvarsan in the Treatment of Acute Osteomyelitis and Blood Stream Infections Caused by the Staphylococcus Aureus, West. J. Surg. 44: 655, 1936.
LeCocq, J.: Personal communication.
Unpublished data.
3a. Osgood, E. E.: Neoarsphenamine Therapy of Bacterial Infections, Arch. Int. Med. 69: 746, 1942.
b. Unpublished data.
4. Osgood, E. E., and Brownlee, Inez E.: Culture of Human Marrow: Details of a Simple Method, J. A. M. A. 108: 1793, 1937.
Osgood, E. E.: The Culture of Human Marrow as an Aid in the Evaluation of Therapeutic Agents, J. Lab. & Clin. Med. 24: 954, 1939.
5. Osgood, E. E., and Ashworth, Clarice M.: Atlas of Hematology, San Francisco, 1937, J. W. Stacey, Inc., pp. 205-206.
Osgood, E. E.: A Textbook of Laboratory Diagnosis, Philadelphia, 1940, The Blakiston Co., pp. 499-500.
6. References 1b and 3a.
7. Osgood, E. E.: The Comparative Effectiveness of Neoarsphenamine, Sulfathiazole, Sulfadiazine, Sulfapyridine, Sulfanilamide, and Other Sulfonamide Drugs Against Neisseria Gonorrhoeae in Marrow Cultures, Am. J. Syph., Gonor. & Ven. Dis. 26: 133, 1942.
8. Moore, J. E.: The Modern Treatment of Syphilis, ed. 2, Springfield, 1941, Charles C Thomas, p. 76.
9. Leifer, W., Chargin, L., and Hyman, H. T.: Massive Dose Arsenotherapy of Early Syphilis by Intravenous Drip Method: Recapitulation of the Data (1933 to 1941), J. A. M. A. 117: 1154, 1941.

THE EVALUATION OF ANTISEPTICS AND OTHER ANTI-INFECTIOUS AGENTS*

Ward J. MacNeal, M.D., and Nancy C. Farnsworth, B.S.
New York, N. Y.

COMMUNICABLE diseases and wound infections have assumed an increased interest because of the continuing extension of belligerent activity in the world and the measures for control of these disorders have again become of primary importance in medicine. One group of agents useful for this purpose includes those which may be employed to kill or inactivate disease-producing microbes or to restrict their activity so as to render them wholly or relatively harmless. Although not quite exact in application, the term antiseptics is used in a broad sense to designate the agents in this category and in this sense the germicides are included among the antiseptics.

Antimicrobic agents may be applied in various ways for the control of infectious diseases and, in general, they may be roughly grouped in three classes according to locus of action. Those which are applied to the microbe while it is still at a distance from its possible new victim are in the first category. Methods for sanitary disposal of human wastes, especially incineration, and the adequate protection of water and food supplies belong here. The boiling of drinking water, as in the preparation of tea in China, and the general chlorination of potable waters elsewhere rank high as hygienic measures. By the time the water is actually ingested by man the dangerously destructive high temperature or high concentration of chlorine is no longer present. These agents perform their beneficent function and then become diluted, chemically combined or otherwise vitiated before reaching the human body. If, however, they still retain their death-dealing properties at this time, the possible danger to man requires attention. Food sterilized by heat may be quite acceptable while that preserved with mercuric chloride or formaldehyde is not. Much of our knowledge of these protective measures belongs in the realm of rather ancient history[1] and the practical applications in sanitation have become routine. However, there still remain unsolved problems in this field and further exact information as to the existence and distribution of pathogenic agents outside the human body may lead to new practical advances. The evident distribution of the virus of poliomyelitis in sewage[2] and by con-

*From the Department of Bacteriology, New York Post-Graduate Medical School and Hospital, Columbia University.

Aided by a grant from the Lambert Pharmacal Company, St. Louis, Mo.

Presented in part before the Joint Session, New York City and New Jersey State Branches, Society of American Bacteriologists, New York City, Dec. 29, 1942.

Received for publication, Sept. 24, 1942.

taminated insects[3] and the animal reservoirs of the virus of yellow fever[4] may be mentioned as items presenting new opportunities in this field.

In the second category may be placed those agents and procedures designed to protect from infection by their application to the surfaces of the human body, the skin, mucous membranes or recently contaminated open wounds. Carbolic acid, mercuric chloride, oxidizing agents and various alcoholic preparations are thus used for their local action as distinct from effect after absorption. The revolutionary advances in antiseptic and aseptic surgery which resulted from the epoch-making observations of Lister were initiated by the use of these agents and no adequately informed person could fail to acknowledge their real value when properly employed. These chemical substances are general protoplasmic poisons, able in some instances to destroy human tissues and even to kill the patient if administered internally in sufficient amounts. Hence they are used for their *local action* in such a way that the amount absorbed is too small to induce dangerous poisoning. This fundamental concept, very clearly understood in the preceding century, has become somewhat obscured by the teachings of some modern authors who have failed to distinguish clearly between the *local* protective action of an antiseptic and the effect of an antimicrobic agent *after absorption*. Garrod[5] has expressed his astonishment that this distinction has recently been so lightly disregarded. He has emphasized that local antiseptics help in cleansing the wound, deodorize it, prevent access of further infection and prevent transmission of infection to others; during the first two hours, when the infectious microbes are largely on the surface of a wound (contaminated wound) the local antiseptic may suffice to destroy them. The superficial tissues may be injured or even devitalized and the local leucocytes inactivated by this antiseptic procedure but this local destructive action is temporary and is a small price to pay in consideration of the control of the infectious agent. After a wound has been infected for several hours the microbes may have penetrated well beneath the surface where they cannot be effectively reached by the local antiseptic agent.

In determining the suitability of an antiseptic for local application, the experimental animal with a controlled wound should receive first consideration as a criterion. Russell and Falconer[6] made experimental wounds of the brain in rabbits and inoculated these wounds with streptococci, pneumococci or diphtheria bacilli and then, after lapse of an hour, they applied the antiseptic agent to be tested. Some local injury to the delicate brain tissues was to be expected. However, they succeeded in overcoming the infection and obtained complete recovery of their treated animals in contrast to the fatal outcome in untreated controls, by use of proflavine sulfate and 2:7 diaminoacridine hydrochloride. Such *in vivo* studies of antiseptics require considerable technical skill. Less exacting are many of the tests *in vitro*.

The official standard tests of the Food and Drugs Administration, U. S. Department of Agriculture[7] are relatively more simple. Here only one bacterial organism (*Staphylococcus aureus*) is used and its resistance is checked against carbolic acid. These tests are not considered adequate in all instances

as indicated by the following passage in the officially approved publication[7]: "The limitations of the phenol coefficient make it necessary in some cases to judge the germicidal preparation by other tests or by additional tests." These official procedures do afford evidence to decide whether or not the particular antiseptic is capable of killing or inhibiting the staphylococcus under the standard conditions and these procedures have been extensively employed in testing various antiseptic agents intended for local use.

The practice of using any one simple *in vitro* test as a criterion merits a few words of caution. The point of view is unfortunate. It is much better to make a more complete study of the antimicrobic agent to learn as fully as possible its properties, mode of action, limitations and its possible injurious effects upon vertebrate tissues and, from the results of such studies, to arrive at an estimate of its possibilities for good and harm. Actually one may not justly assume that the behavior of *Staphylococcus aureus* or of any other single species of organism toward an antimicrobic agent is a reliable measure of the behavior of other infectious agents. Furthermore, there is no justification for the assumption that any single technical procedure, *in vitro*, should be accepted as a final criterion for evaluating the antiseptic activity against even the single organism tested. A variation of technique may bring a new revelation. Therefore, new technical procedures should be welcomed as means of extending our knowledge but certainly they should not pretend to supersede the older "standard" methods. If one sets out to devise a single test to extol or to discredit any particular antiseptic, his task is not difficult provided other evidence can be excluded from consideration.

A brief consideration of some common antimicrobic agents may serve to illustrate the pitfalls and to indicate the many factors requiring attention. Mercuric chloride in a 1:1,000 dilution kills within one minute the staphylococcus in a not too heavy watery suspension. However, if the bacteria are suspended in blood or other albuminous fluid, this germicidal effect may be considerably delayed or even prevented. Furthermore, a new element of difficulty is thus encountered because it may not be easy to decide whether or not the microbe has been killed. Transfer to a medium containing ammonium sulfide or *inoculation into the living animal* may reveal continuing viability of the microbe although the ordinary transfer culture may remain inhibited by the associated trace of the germicide. Local tissue injury caused by mercuric chloride may ordinarily appear unimportant but if this agent is applied to large body surfaces for considerable periods it may be absorbed in sufficient amounts so as to induce severe nephritis and fatal anuria.

Iodine, especially in alcoholic solution, has been a favored local antiseptic for many years. It stains the superficial elements of the skin or mucous membrane or wound surfaces, if these are not too moist. The iodine remains for a time in the stained tissue, being more rapidly removed where there is abundant moisture and active circulation of blood. While the tissues are stained with iodine, the bacteria do not grow in them, but by removal of the iodine with thiosulfate one may often discover that the bacteria have not been exterminated

but are only in a dormant state. Hence in testing the germicidal action of iodine, the neglect of this step to inactivate the residual iodine may permit misleading results.

The soaps are only mildly antagonistic to ordinary bacteria but they have long held a favored place in the hygienic care of the skin, accessible mucous membranes and recent superficial wounds, largely because they aid in the mechanical removal of exfoliated epithelium, decomposing secretions, extraneous material and microbes. Practical medical and surgical experience has long justified the confidence placed in soaps. More recently it has been shown[8] that soaps possess remarkable potency to inactivate certain of the disease viruses, such as those which cause influenza, herpes and poliomyelitis. Obviously soaps are used for their local action, and their destructive effect upon blood[9] or internal tissue elements is of academic rather than practical importance in the antiseptic field.

One of the least dangerous of local antiseptics in general use is represented by liquor antisepticus of the National Formulary[10] and the many commercial preparations of similar composition. These are essentially mixtures of dilute alcohol, thymol, boric acid, essential oils and aromatic organic substances, sometimes with added flavoring and coloring matters. Liquor antisepticus is dispensed in a form so adjusted that it should be employed in the undiluted state and when employed as directed it is able to kill staphylococci promptly. Tests against the viruses[11] of cowpox and of influenza have recently shown that these pathogenic agents are quickly inactivated by liquor antisepticus in concentrations above 80 per cent. The germicidal power of this agent is lost when it is diluted to 50 per cent or more and tests performed with such dilutions are likely to mislead.

In the third category of anti-microbic agents we may consider those designed to act *after absorption,* upon microbes which have already penetrated to the internal medium of the living host, where they are surrounded by lymph or blood or by the protoplasm of living cells deep beneath the epithelial surfaces. Previous to the present century this category was made up almost exclusively of biological products, particularly the antisera produced by procedures of immunization, but in addition there were the purified chemicals extracted from cinchona bark, active against malarial parasites, and emetine, active against the dysenteric amoeba. The modern era of enthusiastic chemotherapy in this field began with the dyes, trypan-rot and trypan-blau, which could be given to mice in doses large enough to tint the entire body and, marvelous to tell, in doses adequate to cause, in the living mouse, the sudden and complete disappearance of trypanosomes from the blood, which had been swarming with these parasites before treatment. This achievement of the Ehrlich School was soon followed by the discovery of the arsphenamines, which could be given to rats in doses sufficient to cause a similar sudden disappearance of the spirochetes of relapsing fever previously swarming in the blood. Here, then, were examples of synthetic chemicals capable of selective destruction of certain microbes without, at the same time, killing the animal host. Subsequently various dye substances and many other synthetic chemicals have

been introduced and exploited. The enthusiastic reception of these agents has been due, in part at least, to their demonstrated actual efficiency. The most spectacular of recent antimicrobic chemotherapeutic agents are represented by the sulfonamides. It is possible to introduce sulfanilamide into mice by stomach tube or by parenteral injection in sufficient quantity to overcome an otherwise fatal infection with hemolytic streptococci and abundant clinical trial has demonstrated the value of sulfonamides in human infections with bacteria of this type and also of other types, especially gonococcus, pneumococcus, some types of staphylococcus, colon bacilli and sporogenic anaerobes. The continuing exploitation of new kinds of sulfonamides, which appear to be well-nigh inexhaustible in variety, affords opportunity for continued renewal of enthusiastic expectations and the febrile patient as well as his physician is often tempted to use one of these drugs even before the microbic cause of the fever has been ascertained, sometimes with disastrous result.

In testing these drugs, their administration to animals infected with the particular microbe was the first and still remains the basic and most reliable criterion of efficiency. Testtube methods are less expensive. One may determine the drug concentration necessary to prevent or restrict the growth of the particular bacteria in a culture medium such as broth, agar or citrated blood. When this is carefully done some interesting observations emerge, showing that biological study of this sort is not so simple as might at first be assumed. Prevention of bacterial growth has been found to depend not alone on the concentration of the drug in the medium but also to an important degree upon the size of the bacterial inoculum. Thus, when less than 1000 streptococci have been seeded, their growth has been completely suppressed by sulfanilamide in a concentration of 1 to 100,000, while with an inoculum of a million or more streptococci growth goes forward even in a drug concentration of 1 to 100. Furthermore, it has been found that the addition of a minute amount of para-amino-benzoic acid[12] to the culture medium serves to overcome the bacteriostatic influence of the sulfonamide and thus to permit growth even of a small inoculum. Other substances not yet precisely identified exhibit a similar influence.

Very ingenious technical methods have been employed by Fleming[13] to demonstrate and to test the antibacterial effects of the sulfonamides and of some other antiseptics. He uses slide cells in which citrated blood inoculated with variable small numbers of hemolytic streptococci and mixed with variable small amounts of drug are sealed in with sterile vaseline paper strips and incubated. Bacterial growth is grossly revealed by hemolysis and can be recorded by photography. When freshly prepared, the citrated blood contains living leucocytes and the conditions approximate those of the circulating blood. A small inoculum of less than ten streptococci may be completely suppressed by the natural citrated blood itself without addition of drug, whereas the same citrated blood after passage through cotton to remove the leucocytes will permit the streptococci to grow. Even more interesting, it was found that the addition of minute amounts of antiseptics, as, for example, carbolic acid 1 part in 700 of blood, serves to inhibit the natural anti-bacterial activity of the

phagocytic cells so that the minute inoculum of streptococci is permitted to grow and to hemolyze the blood while the control preparation without phenol shows no growth. Of course, when a sufficient strength of the phenol is used the growth is suppressed. Fleming has used this method to ascertain the effects in blood of very minute concentrations of various older antiseptics as well as of the newer sulfonamides and also to demonstrate the importance of the size of the inoculum and the variable behaviors of different kinds of bacteria. In another technical procedure, Fleming[13] has used solid agar in a Petri dish. Decimal dilutions of the bacterial culture are spread as broad streaks across the dish on the surface of the medium. Then a segment of the solid agar is cut out at one side of the dish and the resulting defect is filled with melted agar containing the drug to be tested, for example, 1 to 300 sulfanilamide. Diffusion of the drug extends from the insert into the original agar and tends to inhibit bacterial growth to a variable distance from the line of junction and by observation of this inhibition one may estimate the relative susceptibility or resistance of the particular bacterial strain to the drug and may also observe the importance of size of inoculum in determining the growth. This procedure is perhaps less laborious and exacting than the blood-slide culture method and it lends itself to the prompt testing of a culture recently isolated from the patient. These studies of Fleming help toward a better understanding of the action of chemotherapeutic agents in the blood stream and in the internal tissues, *after absorption*.

Somewhat analogous comparative studies of tissue toxicity and antibacterial influence have been carried out by other investigators. Osgood[14] has used *in vitro* tissue cultures of the cells of bone marrow to ascertain the quantity of drug required to inhibit bacterial growth in such cultures and the quantity of drug which will bring about destruction of the tissue cells, thus determining the effective antibacterial concentration and the effective tissue-toxicity concentration.

More recently Welch[9] and his associates have attempted to estimate toxicity of antibacterial agents by microscopic observation of phagocytic activity of leucocytes in diluted citrated blood to which the drug is added and to compare this tissue toxicity with the germicidal effect of the drug in a somewhat different dilute citrated blood.

We have used technical methods of Fleming and those of Welch in the study of various chemotherapeutic agents and have been able to confirm the observations of Fleming in regard to the sulfonamides. By the technic of Welch, however, it was found that azosulfamide is "toxic" in a 1:100 dilution and "not germicidal" in any concentration applicable by the prescribed technique and hence having an immeasurable "toxic index" above 40. Precisely analogous results were obtained with sodium sulfapyridine. Unfortunately, Welch and his associates have attempted to utilize this technical procedure, employing citrated blood, as a criterion for evaluation of antibacterial agents designed for *local use* on the body surfaces, an application which, as has been so clearly indicated by Garrod, is unscientific and likely to be misleading. In

our laboratory a test, by the technique of Welch, of such substances as lemon juice, whiskey, clear tea infusion, black coffee infusion, grapefruit juice, vinegar, tomato juice and baking soda, has shown that each of these agents exercises, according to this technical procedure, a powerful "toxic" inhibitory influence upon the phagocytic activity of leucocytes, while they are without demonstrable germicidal value.

The "toxicity" tests upon tissue cultures or upon fresh citrated blood have real value in the study of antibacterial agents acting *after absorption*. They are not, however, entirely adequate and reliable and may fail completely to disclose the important danger. The animal body includes different kinds of tissue elements which vary among themselves in their reactions to drugs. A concentration of alcohol too weak to influence the cells in a tissue culture or to inhibit leucocytes in citrated blood, may, nevertheless, when present in the circulating blood, cause alterations in the living nervous system which result in traffic accidents. Mercuric chloride in the blood in a concentration too small to influence phagocytosis may cause death from nephritis and uremia. Sulfamethylthiazole maintained in the blood at a level without demonstrable effect on leucocytes or tissue cultures may induce enduring paralysis. Sulfapyridine and sulfathiazole cause toxic injury, not by inhibiting phagocytosis, but by lesions in kidneys, gastrointestinal irritation, changes in bone marrow, myocardial injury[15] and other more obscure alterations. Deaths[16] directly ascribed to administration of the sulfonamides have recently been mentioned in medical publications. To those familiar with the methods of pharmacologic science, these comments will appear superfluous, but they seem to be needed at this time.

In our laboratory an attempt is being made to study tissue toxicity upon the *entire animal* by the relatively inexpensive procedure of injecting the drug into embryonated eggs.[17] The developing chick is a fairly standardized test object and the injected drug does not escape from the shell before hatching. Death of the embryo or survival and successful hatching are grossly evident. The method lends itself to comparative tests of antimicrobic agents alone, of these same agents mixed with infectious viruses or bacteria and of the infectious agents alone. It may prove useful.

SUMMARY

A distinction has been drawn between three groups of antimicrobial agents according to locus of action: (1) those acting apart from the human host called disinfectants; (2) those acting on the epithelial surfaces or upon superficial wounds, designated as antiseptics; and (3) those acting after absorption and classed as anti-infectious therapeutic agents, chemical and biological.

There is no single, easy and simple technical method of evaluating all these agents. The method of Ehrlich and Kolle, in which one administers large amounts to animals to ascertain the lethal dose and smaller amounts to infected animals to ascertain the therapeutic dose, approaches most nearly the ideal procedure. Unfortunately, not all microbes can be tested against drugs

in this way and when feasible the experiments are costly and the relationships sometimes so complex as to leave many questions unanswered. Various kinds of studies, including those *in vitro,* are, therefore, to be welcomed. We are opposed to the attempt to evaluate an anti-infectious agent on the basis of a single "standard" test and we must be on guard against the misleading result of a specially devised technique designed to extol or to condemn some particular agent. It is important also to recall that the causes of infectious diseases are many and varied in nature and that the drug effective against a spirochete may not control the anthrax bacillus, nor may the agent effective against the pneumococcus be assumed necessarily to be potent against the virus of small-pox or influenza. Especially unfortunate is the all too common assumption that an "antiseptic" must be considered worthless unless it may be relied upon to control all infectious agents in all concentrations. So far as we know, only incineration may be accorded this degree of efficiency with full confidence.

Biology is a science of complexities and the control of infectious diseases is a biological problem of the first order. We suggest therefore a broad approach to the study of anti-infectious agents, which welcomes all obtainable information from every reliable means of observation. One may thus determine the peculiar properties of the agent studied and may decide as to its place of usefulness, if any, in the control of infectious diseases. Too frequently the lack of this essential knowledge about an antiseptic and its applicability is the actual reason for failure in its use.

CONCLUSIONS

1. Anti-infectious agents acting on microbes at a distance from the host may be designated as disinfectants. Their toxicity for the human host may be unimportant when it is vitiated before they reach the host.

2. Agents applied to the body surfaces to oppose disease germs may be designated as antiseptics. Such agents should exert the desired local effect upon microbes and should be free from danger of serious poisoning, particularly after absorption. Animal experimentation, tests *in vitro* and clinical trial may contribute to the evaluation of such agents.

3. Antimicrobic substances acting after absorption belong to the category of chemical and biological therapeutic agents. The desired effect upon the microbe should be obtained by a drug concentration much less than that required to kill the animal host. Possible toxic effects may endanger particular organs or tissues and may sometimes be revealed only by animal experimentation or clinical trial.

4. It is possible to discredit or to extol a supposed antiseptic by employing a single selected test procedure to the exclusion of other evidence. Such misleading methods are not recommended.

5. Evaluation of an anti-infectious agent should be based upon all available knowledge of its properties obtainable by various procedures.

REFERENCES

1a. MacNeal, Ward J.: The Preservation of Food. Chapters in Microbiology edited by Charles E. Marshall, Philadelphia, 1917, P. Blakiston's Son and Co.
1b. MacNeal, Ward J.: Pathogenic Micro-organisms, Philadelphia, 1920, P. Blakiston's Son and Co.
2. Paul, John R., Trask, James D., and Gard, Sven: II. Poliomyelitic Virus in Urban Sewage, J. Exper. Med. 71: 765-777, 1940.
3. Paul, J. R., Trask, J. D., Bishop, M. B., Melnick, J. L., and Casey, A. E.: The Detection of Poliomyelitis Virus in Flies, Science 94: 395-396, 1941.
4. Bugher, John C., Boshell-Manrique, Jorge, Roca-Garcia, Manuel, and Gilmore, Raymond M.: The Susceptibility to Yellow Fever of the Vertebrates of Eastern Colombia. I. Marsupalia, Am. J. Trop. Med. 21: 309-333, 1941.
5. Garrod, L. P.: Discussion of the Effect of Antiseptics on Wounds, Proc. Roy. Soc. Med. 33: 487-494, 1940.
6. Russell, Dorothy S., and Falconer, Murray A.: Discussion of the Effect of Antiseptics on Wounds, Proc. Roy. Soc. Med. 33: 494-497, 1940.
7. Ruehle, G. L. A., and Brewer, C. M.: United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. Circular No. 198, U. S. Department of Agriculture, Washington, D. C., December, 1931 (quotation from page 10).
8. Burnet, F. M., and Lush, D.: The Action of Certain Surface-Acting Agents on Viruses, Australian J. Exper. Biol. & M. Sc. 18 (Part 2): 141-150, 1940.
9a. Welch, Henry, and Hunter, Albert C.: Method for Determining the Effect of Chemical Antiseptics on Phagocytosis, Am. J. Pub. Health 30: 129-137, 1940.
9b. Welch, Henry, and Brewer, Charles M.: Relative Toxicity of Certain Antiseptics Containing Soap and Alcohol. With Special Reference to Mouth Washes, Am. J. Pub. Health 32: 261-267, 1942.
9c. Welch, Henry, Slocum, Glen G., and Hunter, Albert C.: Method for Determining the Toxicity of Antiseptics as Measured by the Destruction of Human Leukocytes, J. Lab. & Clin. Med. 27: 1432-1438, 1942.
10. National Formulary, ed. 4, Official from September 1, 1916, American Pharmaceutical Association, Philadelphia, 1918, J. B. Lippincott Co.
11. Dunham, Wolcott B., and MacNeal, Ward J.: Inactivation of Influenza Virus by Mild Antiseptics, J. Bact. 43: 85, 1942.
12a. Woods, D. D.: Relation of p-amino-benzoic Acid to Mechanism of Action of Sulfanilamide, Brit. J. Exper. Path. 21: 74-90, 1940.
12b. Wood, W. Barry, and Austrian, Robert: Studies on the Anti-bacterial Action of the Sulfonamide Drugs. II. The Possible Relation of Drug Activity to Substances Other Than p-amino-benzoic Acid, J. Exper. Med. 75: 383-394, 1942.
13. Fleming, Alexander: Antiseptics and Chemotherapy. Section of Odontology, Proc. Roy. Soc. Med. 33: 127-136, 1939-1940.
Fleming, Alexander: Discussion of the Effect of Antiseptics on Wounds, Proc. Roy. Soc. Med. 33: 487-494, 1940.
14a. Osgood, Edwin E.: Culture of Human Marrow: Studies of the Relative Effectiveness of Neoarsphenamine, Mapharsen, Sulfanilamide, Sulfapyridine, Sulfathiazole, and Sulfamethylthiazole on Infections With Streptococcus viridans (Alpha Hemolytic Streptococcus), Am. J. M. Sc. 200: 596-603, 1940.
14b. Osgood, Edwin E.: Neoarsphenamine Therapy of Bacterial Infections, Arch. Int. Med. 69: 746-765, 1942.
15. French, A. J., and Weller, C. V.: Interstitial Myocarditis Following the Clinical and Experimental Use of Sulfonamide Drugs, Am. J. Path. 18: 109-118, 1942.
16. Lederer, Max, and Rosenblatt, Philip: Death During Sulfathiazole Therapy. Pathological and Clinical Observations on Four Cases With Autopsies, J. A. M. A. 119: 8-18, 1942.
17. Dunham, Wolcott B.: Toxicity of Antiseptics for the Chick Embryo, Proc. Soc. Exper. Biol. & Med. 50: 274-276, 1942.

SPONTANEOUS RUPTURE OF THE SPLEEN DUE TO ACUTE LEUCEMIA or ACUTE LEUCEMIA DUE TO TRAUMA TO THE SPLEEN . . . WHICH?*

REPORT OF CASE AND REVIEW OF THE LITERATURE

A. S. RUBNITZ, M.D., OMAHA, NEB.

WITH THE COLLABORATION OF E. B. FLOERSCH, M.D., COUNCIL BLUFFS, IOWA

SPONTANEOUS rupture of the spleen in leucemia is a rare occurrence. A review of the literature revealed only seven such possible cases. Hannesfahr[1] (1923) states: "Unusually rare are spontaneous ruptures (of the spleen) in leucemia, of which there are only two cases in the literature, described by Rokitansky (1861)." Lange[2] (1928) mentions five cases from the literature and adds one of his own. Only one more case has been recorded since 1928, that of Neal[3] (1940).

As to the relation of trauma to leucemia, Olovson[4] (1939) collected 67 cases from the literature, and added two of his own, in which leucemia happened to occur in association with trauma to the spleen or to long bones. Six more cases have been reported since Olovson's paper was published: three by Yaguda and Rosenthal[5] (1939), two by Conzett[6] (1939) and one by Schau[7] (1939). There was also one case reported by Novikoff (1933) (cited by Forkner[8]) which Olovson failed to include in his tabulation, so that altogether about 76 such cases have been recorded. While it is true that in many of the reported cases the authors did not claim that trauma was responsible for the leucemia, yet a sizable number of cases have accumulated in the literature in which an etiologic relationship between the two seemed to exist.

The case presented here is of interest primarily because of the rarity of spontaneous rupture of the spleen in leucemia and secondarily because it brings up for consideration the question of traumatic leucemia.

CASE REPORT

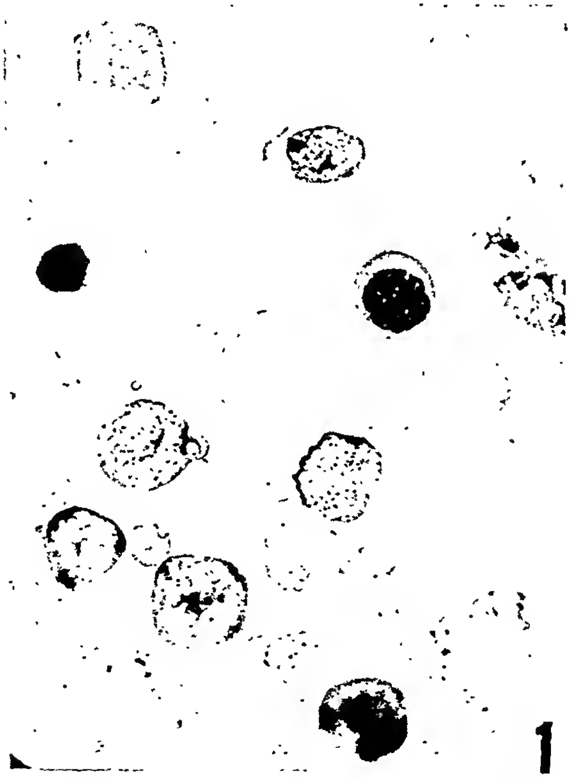
H. B., white male, aged 24, milk delivery-man, was admitted to the Mercy Hospital on January 31, 1941. Chief complaints: weakness, abdominal cramps, nausea, profuse sweating and loss of weight. Family history was unimportant. Past history: at the age of 11 he was operated upon for appendicitis complicated by peritonitis. At the age of 13 he suffered a severe injury to the left side of the abdomen and the left flank, in a coasting accident; this was followed by hematuria. The physician who attended him at that time expressed the belief that both the left kidney and the spleen were ruptured. However, the hematuria quickly subsided, and the patient recovered from the accident in a relatively short time, seemingly suffering no ill effects subsequently.

History of present illness: On December 22, 1940, while the patient attempted to climb on the wagon in the course of his daily work, the horse started unexpectedly with a

*From the Department of Medicine, University of Nebraska, Omaha, and the Department of Pathology, Mercy Hospital, Council Bluffs, Iowa.

Received for publication, Sept. 28, 1942.

jerk; the patient missed the step and fell to the pavement twisting his body and the lower back. Upon examination at his home within three hours after the accident, skin abrasions over the right elbow were the only visible marks of injury. The objective findings were those of "lumbo-sacral sprain," and the patient was taped with adhesive. At the physician's office the following day, x-ray examination of the back and pelvis showed no fractures. Patient returned to work in a few days. About a week later he came down with the "flu" (as diagnosed by the attending physician). In a few days he again returned to work, but complained of being easily fatigued. On January 25, 1941, he reported for a physical examination, expressing the belief that he was suffering from "stomach ulcers." A routine blood count was done; the hemoglobin was 75 per cent; the erythrocytes numbered 4,200,000 and the leucocytes 9,800 per c.mm. Examination of the blood smears was not made. He was advised to go to the hospital for observation. This he refused to do at first, but consented to a few days later (Jan. 31).



1

Fig. 1.—Photomicrograph of blood smear taken on Feb. 13. ×930.

Upon entrance to the hospital, his temperature was 102° F.; there was abdominal tenderness and rigidity, and slight cervical adenopathy. The blood count was: Hgb. 14 grams; red blood cells, 4,640,000; white blood cells, 21,500. The differential count (as recorded by the technician) was: segmented neutrophiles 8 per cent; eosinophiles 1 per cent; lymphocytes (?) 91 per cent.

Progress in the Hospital.—The leucocyte count was 35,000 on Feb. 3. A careful study of the blood smears (A. S. R.) disclosed that almost 80 per cent of the leucocytes were promyelocytes, with occasional blast-cells. Most of the cells were oxydase positive. These findings were suggestive of acute leucemia. The clinical picture, however, did not warrant this diagnosis: there were no purpuric manifestations, no ulcerative lesions in the mouth, and but slight swelling in the cervical lymph glands. The patient's chief complaint was abdominal pain, not limited to any particular region. On account of overlying

muscle guard the spleen could not be palpated. The leucocyte count continued to rise: on Feb. 4 it was 40,700; on Feb. 7, 68,400, and on Feb. 11, 82,100. The appearance of the cells in the stained smears was frankly leucemic. In the last smear (see Figs. 1 and 2) 88 per cent of the leucocytes belonged to the young myelocytic series, mainly promyelocytes with occasional blast-cells and rare myelocytes. Nucleated red cells became very frequent. The red count on Feb. 11 was 3,600,000 and the hemoglobin 10.2 grams. A diagnosis of acute leucemia was made and a poor prognosis given. In spite of this the patient did not appear to be critically ill.

Fig. 2.—Photomicrograph of blood smear taken on Feb. 13. ×1700.

Other laboratory findings: (1) The urine contained a trace of albumin, isolated leucocytes and rare granular casts. (2) The blood N.P.N. was 36 mg. per cent and the creatinine 1.4 mg. per cent. (3) The hetrophile antibody reaction was negative. (4) The icterus index was 5. (5) The blood culture remained sterile at the end of 72 hours.

On Feb. 14 the patient, feeling unable to eat his breakfast, "forced down some food" and vomited shortly after. About 10 A.M. he became very restless and complained of weakness and pain in the precordium, requiring two hypodermic injections of morphine during the day for relief. He dozed for short intervals but continued to complain of precordial pain. His respirations became labored. At 6 P.M. his temperature rose to 104°, his pulse could not be counted accurately (recorded as 156?) and the respirations were 42 per min. He became irrational and involuntary. He died at 3 A.M., Feb. 15.

Autopsy was done at 8 A.M. Feb. 15. The body had been embalmed but the abdominal cavity had not been disturbed.

Important gross findings, as abstracted from the autopsy protocol: The left pleural cavity contained over one quart of thin blood-tinged fluid. The pericardial sac contained about 100 c.c. of bloody fluid. There were some ecchymotic areas in the epicardium near

the apex. The peritoneal cavity contained over one quart of clotted blood. A mass of confluent glands, the size of a hen's egg, was present in the mesentery of the ileum near the cecal juncture. The entire root of the mesentery was infiltrated with large glands, many of them the size of a walnut (see Fig. 4). The retroperitoneal lymph nodes along the aorta were greatly enlarged and formed a continuous chain extending from the pelvis to the diaphragm. The left kidney was rather small, weighing 110 grams; it was round

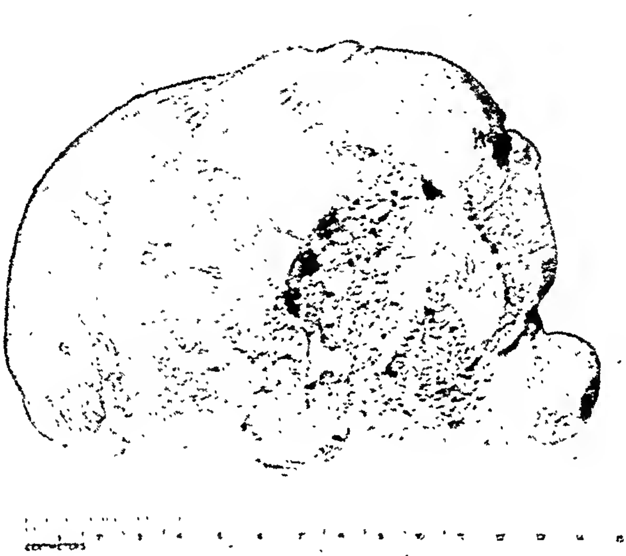


Fig. 3.—Photograph of ruptured spleen.

Fig. 4.—Photograph of mass of glands in the root of the mesentery.

and flat; the calices were distorted. The right kidney was of normal shape and quite large, weighing 330 grams. The spleen was enveloped in a mass of omentum. As the omentum was shelled off, a very soft, enlarged ruptured spleen was found. The rupture occurred almost through its middle, transverse to the long axis, and extended from the concave border nearly halfway across the width of the organ (see Fig. 3). The spleen weighed 420

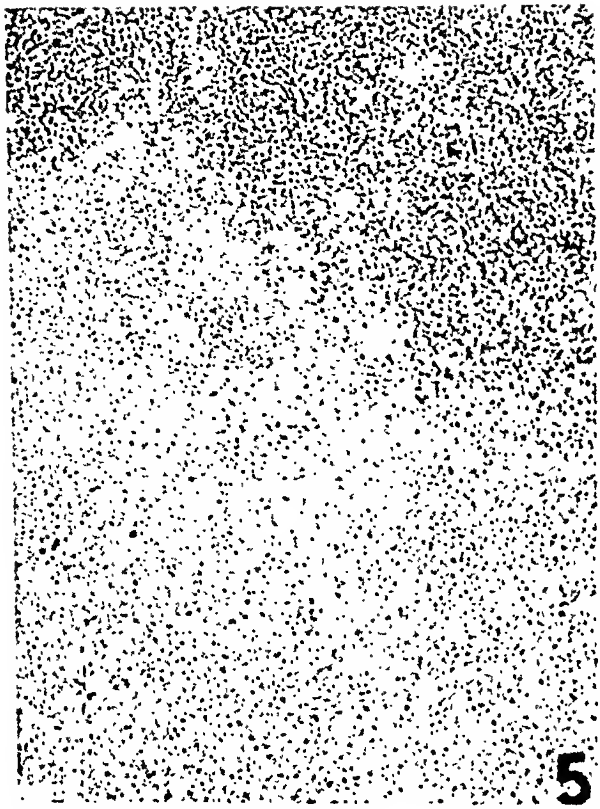


Fig. 5.—Photomicrograph of section of the spleen. ×140.

Fig. 6.—Photomicrograph of section of the liver. ×140.

grams; the tissue was unusually soft. On section, the color varied from reddish-brown to dark-chocolate. Many areas were cystic, the tissue being in a fluid state. The liver weighed 2390 grams. The cut surface was very soft. Both lungs had dark patches at the bases. On section, large edematous areas were present through the posterior portions of both lungs, especially in the lower lobes.

Summary of the gross anatomic findings: (1) Ruptured, degenerated spleen; (2) massive intraperitoneal hemorrhage; (3) swelling of the mesenteric and retroperitoneal lymph nodes; (4) left hydrothorax; (5) hemopericardium; (6) distortion and scarring of the left kidney and compensatory hypertrophy of the right kidney; (7) hypertrophy and degeneration of the liver; (8) pulmonary edema.

Important microscopic findings: Sections of the lymph nodes presented the usual histologic picture of leucemia. Frank leucemic changes were also evident in sections of the spleen, though here the process of necrosis overshadowed all other findings (see Fig. 5). Sections of the liver showed generalized parenchymal necrosis, fatty metamorphosis, and areas of leucemic infiltration. The latter were most pronounced in the interlobular connective tissue, around the ducts (see Fig. 6). Leucemic infiltration was also apparent in sections of the pancreas, kidneys, adrenals and lungs.

COMMENT ON CASE

It is difficult to evaluate the history of injury at the age of 13. According to the physician who attended him at that time, the patient had a hematuria which cleared up in a few days. Rupture of the spleen was suspected at first but the patient seemed to be in good physical condition and never manifested any evidence of internal hemorrhage. Moreover, by the time the hematuria disappeared, he seemed to have totally recovered from the effects of the injury.

The history of the accident six weeks before the patient's entry to the hospital permits a good deal of speculative reasoning.

One could assume that the patient suffered a subcapsular tear in the spleen, and that a hematoma was formed. The patient continued to complain of abdominal discomfort and developed fever and malaise which was diagnosed as "the flu." The hematoma could lead to increased tension within the spleen and cause degenerative changes in the pulp. All through the period of illness the degeneration might have continued to progress, and finally the organ might have ruptured from a trivial increase in pressure when the patient turned in bed or during the act of vomiting. This line of reasoning does not take into account the leucemic changes in the blood and tissues.

Again, one might speculate that the accident had nothing to do with the patient's illness. The onset of the illness might have happened to coincide with the time he returned to work after the accident. His complaints of malaise, weakness, anorexia and abdominal cramps were diagnosed as "the flu," a diagnosis often made at the onset of acute leucemia. The blood findings were not characteristically diagnostic at first but became more and more so as the disease progressed. simultaneously with the rapidly changing blood picture. and culminated in sudden spontaneous rupture.

A third combination could be rationalized: it might be assumed that the recent accident was not severe enough to produce rupture in a normal spleen. However, the accident suffered by the patient at the age of 13 might have produced a tear in the spleen which had healed. The scar formed in the line of healing might have given way during the last accident and brought about all

the sequelae of the first mentioned possibility. The fact that the spleen was highly degenerated made it impossible to demonstrate any preexisting lesions in this organ. From the appearance of the left kidney, however, one could surmise that the distortion in its shape was due to the coasting accident and that the spleen quite likely also suffered injury at that time.

GENERAL DISCUSSION

Spontaneous rupture of the spleen is invariably associated with preexisting disease of this organ. A normal spleen will not rupture unless it is subjected to violence of a considerable degree. Rupture of the spleen in the absence of obvious trauma is an indication that deep-seated degenerative and necrotic changes have taken place. Necroses of the spleen in leucemia are not uncommon. According to Lubarsch (cited by Schultz[9]), they occur in up to 50 per cent of the cases of myeloid leucemia, and are supposedly due to arterial thromboses. The damage of the arterial wall may be similar to that produced in periarteritis nodosa which leads to the formation of thrombi. According to Benda (cited by Schultz), the infiltration of the vascular wall with leucemic cells is a common occurrence in leucemia. Such infiltration within the walls favors formation of thrombi within the lumen. Another factor, initiating thrombus formation in leucemia, was pointed out by Hirschfild (cited by Schultz), as being the increased viscosity of leucemic blood. He explained the formation of thrombi in the corpora cavernosa on the same basis.

While the above-mentioned vascular changes produce or tend to produce necrotic changes in the spleen, such necroses are generally of a mild degree. Quite rare are the necroses which involve the greater part of the organ, as in the case recorded by Schultz. Even in his case the necrosis did not lead to rupture of the spleen. Our case is unique in that the degeneration of the spleen involved almost the entire organ. Such extreme necrosis has been recorded by Schtscherbatoff (cited by Friesleben[10]) in a patient who was recovering from a severe case of typhoid fever and who after a very light strain suddenly collapsed and died. Autopsy disclosed a transverse rupture of a totally disintegrated spleen.

The sequence of events in our case could be viewed from two different angles: (1) This was a case of rapidly progressing acute leucemia. The degenerative changes in the spleen paralleled the day-by-day hematologic changes, culminating in total disintegration and rupture of the organ. (2) This was primarily a case of degeneration of the spleen, initiated by a subcapsular hematoma of traumatic origin. The leucemic changes in the blood resulted from abnormal stimulation of the hematopoietic apparatus by the disintegrating spleen.

In a paper published under the title "Leukemia Following Trauma," Lewsen (1930)[11] collected sixty cases from the literature, including one case of his own. However, the evidence in twenty of the cases was not convincing and he tabulated only 40 of the cases (all of these are also included in Olovson's tabulation). The type of trauma was classified by Lewsen into three groups: (1) blow to long bones or ribs which may or may not result in a fracture; (2) blow in the region of the spleen, sometimes rupturing that organ, and (3) a general severe shaking. Lewsen concedes that trauma to

hematopoietic tissue does not always result in leucemia. He is of the opinion that leucemia would be produced only in individuals who have an inherent predisposition to this disease. He draws an analogy between traumatic leucemia and traumatic sarcoma, pointing out that trauma alone, without the individual predisposition, would hardly be sufficient cause for the production of either.

Analyzing his own tabulated cases, Olovson concludes that they could be divided into 3 groups: (1) those in which trauma leads to the discovery of an existing leucemia; (2) those where trauma causes an aggravation of an existing leucemia, and (3) those where trauma seemingly causes the leucemia. It is obvious that our attention would be centered principally on the cases of group three. Yet, even in these, according to Olovson, trauma could play but a minor role in the production of the disease, because traumata occur frequently whereas traumatic leucemia is a rare occurrence. He is of the opinion that trauma may constitute the exciting factor in a person predisposed to leucemia.

Yuguda and Rosenthal are still more outspoken in their view that trauma could not be the sole factor in the production of leucemia. These authors believe "that trauma initiates leucemia in a person with a preexisting tendency to the disease. The injury upsets the fine hematopoietic balance which has maintained the leucemia in its latent state and causes a more rapid progression of the process with development of frank symptoms of leucemia."

In the main all the above authors agree that trauma to hematopoietic tissue by itself could not be considered sufficient cause to produce leucemia. They all believe that trauma plays the role of a precipitating factor, in an individual who has an inherent tendency to the disease.

Mention may be made here that the problem of trauma and leucemia also attracted attention in veterinary medicine. Cases of leucemia following trauma have been observed in a sheep, a horse, and in dogs, as reported by Weber, Wirth, Share-Jones and Jarmai (all these authors are cited by Olovson). Jarmai conducted a series of experiments on dogs in which he traumatized the spleen by an open operation. However, all the experiments resulted in failure to produce any leucemic changes in the animals.

The problem of trauma and leucemia is of special importance from the medicolegal standpoint. In Olovson's series, 19 of the cases have been scrutinized by insurance commissions and 12 were granted compensation. This presumably implies that on the surface, at least, a cause-and-effect relationship seemingly was present in the 12 cases. Furthermore, we should be mindful of the fact that insurance companies will often tend toward leniency in settling an accident claim, particularly where it is known that the individual's illness dated to the accident. None-the-less, insurance companies will not grant compensation unless the case merits consideration.

In order that a case of leucemia could justly be considered as one due to trauma and qualify for compensation, the following criteria were set up by Liniger (cited by Yaguda and Rosenthal):

1. The injured individual must have been well and feeling capable of work up to the time of the accident.

2. A suitably severe accident must have taken place, with the essential subjective and objective signs of injury.

3. The time at which the development of sickness starts and that of the accident must bear a relation, that is, there must be bridging symptoms.

Olovson's criteria are:

1. Good health and absence of disease-symptoms before the trauma.

2. A free interval between the trauma and the first appearance of the symptoms.

3. Leucemia of the myeloid type.

Points 1 in both sets of criteria are essentially identical. Liniger's point 2 does not need any elaboration. Both Liniger's point 3 and Olovson's point 2 deal with the time-element between the trauma and the first manifestations of the disease. However, each one of these authors expresses a different conception and stresses a different point. Both these points merit consideration and can best be emphasized by illustrative cases:

Illustration 1. (Case 2 of Conzette.) A middle-aged woman suffered an intertrochanteric fracture. Upon admission to the hospital, immediately after the accident, her leucocyte count was 188,000 and the blood smear showed the characteristics of chronic myeloid leucemia. The spleen was decidedly enlarged.

Illustration 2. (Case 1 of Olovson.) A 27-year-old male suffered fracture of the leg. The fracture healed and the patient was seemingly in very good health for over 20 months, when he developed pain and swelling at the site of the fracture. At this time his leucocyte count was 347,000 and the blood smear was characteristic of chronic myeloid leucemia.

Of these two illustrations, the first depicts an instance where a leucemic individual suffered a fracture. Olovson's point 2, calling for a free interval between the trauma and the first appearance of the leucemic symptoms, excludes this case from the category of traumatic leucemias, and the case well illustrates the soundness of that point. In the second illustrative case an interval of over 20 months elapsed between the injury and the appearance of leucemic symptoms. There was a total lack of bridging symptoms. Liniger's point 3 would tend to exclude this case from the category of traumatic leucemias.

That the leucemia must be of the myeloid type in order to be considered as of traumatic origin (as advocated by Olovson's point 3) is a strongly debatable question. Nearly all of Olovson's 69 tabulated cases were leucemias of the myeloid type (only 3 of the cases were lymphatic leucemias). These figures, perhaps, were partly responsible for that contention. However, this statistical preponderance of myeloid leucemias is lessened by the fact that out of the 7 cases not included in Olovson's tables, 4 were lymphatic leucemias (one case of Novikoff, one of Schau, one of Yaguda and Rosenthal, and one of Conzette). Discarding the statistical reason for that contention, we come to the more valid reason, namely, that if traumatic leucemia is produced by injury to long bones or to the spleen the implication would be that such injury upsets the myelopoietic tissue, hence leucemia of the myeloid variety only could result. This reasoning is based on the assumption that lymphatic leucemia has its origin in histo-biologic disturbances of the lymphocytic apparatus, whereas myeloid leucemia has its origin in the myeloid tissue. While it is

true that this strict subdivision could be visualized in chronic leucemias, the site of the hematopoietic disturbance in acute leucemias is not as a rule so clearly delimited. The process of metaplasia is a common characteristic of acute leucemia, and tissues which are in no way concerned with the function of hematopoiesis in postnatal life generally revert to their embryonal state and resume such a function, producing abnormal leucocytes of every variety; hence the difficulty in most instances to classify acute leucemias according to cell types. Incidentally, an analysis of all the reported cases of traumatic leucemia reveals that the most authentic cases are those in which acute leucemia resulted from the trauma.

The most weighty argument, however, against Olovson's point 3 is the case reported by Schau:

> An 18-year-old boy was hit in the left side of the abdomen, playing football. At the hospital, rupture of the spleen was suspected but the patient was treated conservatively. The leucocyte count was 9,000. About 7 weeks later the patient was readmitted with a clinical and hematologic picture of frank leucemia; the leucemia, according to the author, was lymphatic in type. Autopsy disclosed an old tear in the spleen.

According to advocated criteria, this case is an excellent example of traumatic leucemia, though it was not one of the myeloid variety.

From the foregoing discussion one may deduct that the criteria advanced by Liniger and by Olovson for the medicolegal evaluation of a case of traumatic leucemia are logical and sound, except point 3 of Olovson in which the author insists that the leucemia must be of the myeloid type.

It might be of interest to mention here that a monetary settlement was granted by the insurance company in our case.

SUMMARY

A rapidly progressing case of acute leucemia is reported. The rapidity of the progress of the disease was most noticeable in the blood changes, whereas clinically the patient did not appear to be critically ill until almost the end. On the fifteenth day of the patient's stay in the hospital his condition suddenly changed for the worse, and after a stormy 18 hour period he expired. Autopsy disclosed a massive intraperitoneal hemorrhage resulting from a ruptured spleen. The spleen was degenerated throughout. The degeneration was attributed to leucemic changes in the organ. However, two previous episodes in the patient's history could have accounted for some damage to his spleen: (1) about 11 years previously the patient was in a coasting accident suffering injury to the left kidney and presumably also to the spleen, and (2) about 8 weeks before his death the patient fell on the pavement, spraining his back and "wrenching" his body. That the patient actually suffered serious injury to the spleen in either of these accidents is only conjectural. Because of the extreme disintegration of the spleen, histologic evidence could not be relied upon to prove or disprove that the organ had suffered injury in the past. It is conceivable that at the time of the coasting accident a tear in the spleen had been produced and that this healed by scar formation. Furthermore, it is conceivable that during the last accident the pulp gave way in the line of the old scar and a subcapsular hematoma was formed, leading to sub-

sequent "spontaneous" rupture. It is granted that no absolute proof exists that the spleen was severely injured in either of the two episodes; yet, because of the fact that the history of the last accident was so intimately tied up with the patient's last illness and because of the patient's persistent complaints of abdominal pain throughout the period of the illness, credence may be given to the view that the degenerative changes in the spleen set in before the hematologic changes were noticeable. Since a number of leucemic cases are recorded in the literature in which trauma to the long bones or to the spleen preceded the leucemic changes, some authors express the belief that leucemia may be precipitated by trauma in susceptible individuals. If we accept the dictum that there is such an entity as traumatic leucemia, our case would fall into that classification, and the entire clinical behavior of the patient, from the day of the accident to the hour of the fatal termination, would appear to be in logical sequence.

The question of the possible causal relationship between the trauma and leucemia is of interest from two angles: (1) the theoretical soundness of the view that leucemia could be caused by trauma, and (2) the medicolegal side of the problem, i.e., should an employer or insurer be held liable for the leucemia in a case where leucemia follows trauma. An attempt has been made in this paper to analyze both phases of the question and to bring out the more authoritative statements and data from the literature regarding this question.

CONCLUSIONS

1. Spontaneous rupture of the spleen in a case of acute leucemia is reported.
2. Since there was a history of an accident suffered by the patient about 6 weeks before the leucemia was established, the case is also discussed as one of possible traumatic leucemia.
3. The literature on the subject of traumatic leucemia is reviewed.
4. The medicolegal criteria recommended for the acceptance of a case as one of traumatic leucemia are scrutinized.

REFERENCES

1. Hammesfahr: Spontanruptur einer leukämischen Milz, Zentralbl. für Chir. 50: 1634, 1923.
2. Lange, W.: Spontanrupturen der Milz bei Leukämie, Med. Klin. 24: 1311, 1928.
3. Neal, J. M.: Rupture of the Spleen in Myelogenous Leukemia, M. Bull. Vet. Admin. 17: 96, 1940.
4. Olovson, T.: Trauma und Leukämie, Acta chir. Scandinav. 82: 63, 1939.
5. Yaguda, A., and Rosenthal, N.: The Relation of Trauma to Leukemia, Am. J. Clin. Path. 9: 311, 1939.
6. Conzett, D. C.: Leukemia Associated With Trauma, J. Iowa M. Soc. 29: 458, 1939.
7. Schau, H.: Lymphatische Leukämie und Milzverletzung, Zentralbl. f. Chir. 66: 1609, 1939.
8. Forkner, C. E.: Leukemia and Allied Disorders, New York, 1938, The Macmillan Co.
9. Schulz, W.: Ueber nicht-embolisch entstandene keilförmige Milznekrosen, Frankfurt. Ztschr. f. Path. 37: 489, 1929.
10. Friesleben, M.: Ein durch Splenektomie geheilter seltner Fall von Spontanruptur der Milz, Deutsche Ztschr. f. Chir. 173: 45, 1922.
11. Lewsen, S. C.: Leukemia Following Trauma, Lancet 1: 288, 1930.

CLINICAL CHEMISTRY

THE EFFECT OF DIATHERMY ON BRAIN METABOLISM: CHANGES PRODUCED ON THE SUGAR, LACTIC ACID, AND pH OF THE ARTERIAL AND VENOUS BLOOD OF THE BRAIN IN PARETIC PATIENTS

JOSEPH M. LOONEY, M.D., AND EMBRIE J. BORKOVIC, M.D., WORCESTER, MASS.

IN A recent communication[1] we pointed out that during diathermy therapy in our patients the oxygen A-V difference showed a fall rather than a rise as obtained by Himwich and his co-workers.[2] In this paper we are reporting the results of hyperthermia on the sugar, lactic acid, and pH of arterial and venous blood. The same procedure for obtaining blood samples was followed as given in the previous paper.[1]

The blood sugar was determined by the method of Folin,[3] the lactic acid by the method of Edwards,[4] and the pH of the whole blood at 38° C. by the glass electrode and an electron tube potentiometer built according to the method of Stadie.[5] All determinations except the blood sugar were made in duplicate. The means and standard deviations for the different variables for the three periods of observations are given in Table I. Each value represents the results obtained from twenty-one separate experiments.

Both the arterial and venous blood sugar levels showed a fall during the period in which the temperature was rising and then an increase to a higher level than the control reading. The decreases in sugar for both venous and arterial blood, as well as the subsequent increases, were statistically highly significant. The difference between the final and initial readings, however, was not significant for either the arterial or the venous blood. The difference between arterial and venous blood sugar increased from a mean of 7.3 mg. for the first reading to 11.0 mg. for the final reading. This change was not statistically significant, as the P value calculated for this change is 0.32, which indicates that this change could occur by chance 32 times in every 100 trials.

The lactic acid levels in both arterial and venous blood showed a slight decrease during the period of elevated temperature. The decrease was greater in the arterial blood, with a fall from 14.22 to 11.97 mg., than in the venous blood, which decreased from 13.36 to 12.15 mg. and then rose to 12.61 mg. As a result of the difference in the degree of change, the final level of lactic acid in venous blood became higher than that of the arterial blood. Although these changes were not statistically significant, the trend is important when considered in connection with the changes which occurred in the oxygen con-

From the Memorial Foundation for Neuro-Endocrine Research and the Medical Service of the Worcester State Hospital, Worcester, Mass.
Received for publication, Oct. 22, 1942.

TABLE I

MEANS AND STANDARD DEVIATIONS FOR ALL VARIABLES FOR THREE PERIODS

		FIRST READING*		SECOND READING*		THIRD READING*	
		MEAN	S. D.	MEAN	S. D.	MEAN	S. D.
Temperature ° F.		98.41	0.700	105.95	0.289	105.92	0.358
Total serum protein (Gm. per 100 c.c.)	Venous	6.60	0.92	6.97	1.00	7.58	1.18
Blood hematocrit	Venous	40.01	3.53	44.42	4.19	43.04	5.12
Specific gravity	Venous	1.0258	0.00174	1.0267	0.00172	1.0283	0.00165
Blood sugar (mg. per 100 c.c.)	Arterial	126.0	34.38	103.8	22.05	140.3	38.95
	Venous	118.8	30.48	94.0	11.29	132.1	37.33
	A-V† difference	7.3	11.43	9.8	18.14	11.0	10.75
Lactic acid (mg. per 100 c.c.)	Arterial	14.22	4.48	12.39	5.08	11.97	4.26
	Venous	13.36	3.88	12.15	5.65	12.61	4.20
	A-V	0.86	2.61	0.24	5.54	-0.64	2.32
pH	Arterial	7.462	0.066	7.514	0.077	7.497	0.072
	Venous	7.398	0.059	7.459	0.068	7.465	0.087
	A-V	0.064	0.033	0.055	0.026	0.037	0.023

*First reading immediately before diathermy; second reading when temperatures reached 106° F., approximately two and one-half hours later; third reading, after three to *four* hours' fever at 106° F.

sumption and sugar metabolism. In our previous paper[1] it was shown that the oxygen consumed decreased as the temperature was maintained at the higher level. This would indicate that the rate of combustion in the brain did not increase with the temperature. The slight increase in utilization of sugar could be explained, at least in part, by the transformation of some of the sugar to lactic acid. The values of lactic acid in the arterial and venous blood of the brain for the first two readings indicate the utilization of some lactic acid by the brain tissues during this period. During the last period, however, there is a reversal, and a production of lactic acid occurs, the venous level being higher than the arterial level.

The pH of the blood showed a significant rise during the period of elevated temperature in both venous and arterial blood. In the arterial blood there was an increase from 7.462 ± 0.0148 to 7.514 ± 0.0172 and then a drop to 7.497 ± 0.0162. The difference between the first and last readings was not statistically significant. In the venous blood the changes from the initial reading, 7.398 ± 0.0132 to 7.459 ± 0.0153 for the second reading and 7.465 ± 0.0152 for the final reading, were highly significant. The values of Fisher's P for the differences between the readings were 0.0023 in the first case and 0.0008 in the second.

The change in the arterial-venous differences in the pH values was highly significant (P = 0.002) between the initial reading of 0.064 ± 0.0072 and the final reading 0.037 ± 0.0051 but was not significant between the first and second readings. With the increase in temperature the difference between venous and arterial blood became smaller, the greatest change occurring between the second and the third readings. The fall in the A-V difference between these readings was entirely accounted for by the fall in the pH of the arterial blood.

DISCUSSION

The elevation in blood sugar has been noted by previous investigators.[6, 7] Our results agree with those of Hench,[6] that the elevation is not due to blood concentration, for we obtained a 40 per cent increase in sugar from the lowest to the highest readings and only a 7.5 per cent hemoconcentration on the basis of the hematocrit readings, or a 14.8 per cent increase on the basis of the total protein levels.

Despite this marked increase in the concentration of sugar in the cerebral circulation, there was no increase in the utilization of sugar. The slight increase in A-V difference noted during the third period could be accounted for, in part, by conversion to lactic acid. These results confirm our previous findings[1] that hyperthermia resulting from diathermy therapy does not increase the rate of brain metabolism.

Our findings also lend support to the view that under certain conditions the brain does utilize lactic acid. However, when the temperature is elevated, lactic acid is produced. This may be due to an interference with the delicately balanced enzyme systems as the result of the increased temperature.

The alkalosis produced (amounting to 0.07 pH units in the venous blood and 0.03 pH units in the arterial blood) is due to the loss of carbon dioxide and is not as marked as that reported by Bischoff and co-workers.[7] These

investigators found increases in plasma pH of 0.12, 0.09, 0.26, and 0.04 in four cases. Cullen, Weir, and Cook[9] in a recent paper indicate pH changes of 2.0 pH units. This value is so high that it seems likely that an error has been made in reporting and that the change should be 0.2 pH units. Their results were obtained on blood taken from the arm, and therefore conclusions as to changes which occur in the cerebral circulation are not warranted.

In the case of one of the patients who died following treatment, the blood sugar showed wide fluctuations in level but no difference between the arterial and venous readings, which were 211 and 211, 95 and 94, and 155 and 154 mg. per 100 c.c. One patient showed a remarkable increase in arterial blood sugar with the following values: 98, 109, and 290 mg. per 100 c.c. It would appear that in this case the continued elevation of temperature for the three-hour interval at 106° F. had caused a marked stimulation of the adrenal mechanism and a release of sugar from the glycogen deposits in the liver. In his second treatment the patient showed an elevation from 95 to 164 mg. Another patient showed a rise from 98 to 190 mg. It is evident that although slight increases in sugar are found in all patients during periods of temperature elevation, very marked changes are the exception. The high mean values for sugar found in the control period are due to the inclusion of two men with abnormally high fasting values. During the period in which active heating of the body was taking place, in fourteen of twenty-one tests the blood sugar fell. This fall also occurred in the two cases that had high initial values, in the first instance from 211 to 95 mg. per 100 c.c., and in the second, from 176 to 78 mg. per 100 c.c. This initial fall in sugar has not, to our knowledge, been reported before.

TABLE II

COEFFICIENTS AND PROBABILITIES FOR CORRELATIONS BETWEEN TEMPERATURE AND INDIVIDUAL VARIABLES

CHANGE IN TEMPERATURE VS.	NO. OF READINGS	R	P
Change in venous blood sugar	38	0.7187	< .01
Change in arterial blood sugar	37	0.5371	< .01
Change in total protein	38	−0.1628	
Change in blood hematocrit	32	−0.0979	
Change in specific gravity	37	−0.2457	
Change in venous lactic acid	38	−0.1516	
Change in arterial lactic acid	38	−0.1119	
Change in venous pH	36	0.5109	< .01
Change in arterial pH	36	0.4725	< .01

The correlations between change in temperature and the other variables are shown in Table II. It will be noted that only those between temperature and sugar and temperature and pH can be considered significant. Contrary to expectation, the correlations for total protein, blood hematocrit, and specific gravity are very low. Incidentally, the correlation between specific gravity and total protein is also low, as pointed out in a paper published recently.[10]

SUMMARY

The changes produced in cerebral blood sugar, lactic acid, and pH by elevation of temperature through diathermy in twelve patients receiving dia-

thermy treatments are given. There was a slight but not significant increase in sugar utilization and a significant increase in pH. The lactic acid A-V difference becomes reversed so that during the period of continued temperature elevation, lactic acid is produced. This would explain, in part, the slight increase in the A-V difference for blood sugar. The findings confirm those previously reported, that there is no increase in brain metabolism during diathermy.

REFERENCES

1. Looney, J. M., and Borkovic, E. J.: Changes Produced on Oxygen and Carbon Dioxide Content of Arterial and Venous Blood of Brain During Diathermy Therapy for General Paresis, Am. J. Physiol. 136: 177, 1942.
2. Himwich, H. E., Bowman, K. M., Goldfarb, W., and Fazekas, J. F.: Cerebral Metabolism During Fever, Science 90: 398, 1939.
3. Folin, O.: The Determination of Blood Sugar, J. Biol. Chem. 82: 83, 1929.
4. Edwards, H. T.: Simplified Estimation of Lactate in Normal Human Blood, J. Biol. Chem. 125: 571, 1938.
5. Stadie, W. C., O'Brien, H., and Laug, E. P.: Determination of the pH of Serum at 38° With the Glass Electrode and an Improved Electron Tube Potentiometer, J. Biol. Chem. 91: 243, 1931.
6. Hench, P. S., and Slocumb, C. H.: Results of Fever Therapy for Gonorrheal Arthritis, Chronic Infectious (Atrophic) Arthritis and Other Forms of "Rheumatism," Proc. Staff Meet., Mayo Clin. 10: 202, 1935.
7. Kerstein, M. B., and Bromberg, L.: Effect of Fever Therapy Upon Carbohydrate Metabolism, J. Lab. & Clin. Med. 25: 7, 1939.
8. Bischoff, F., Long, M. L., and Hill, E.: Studies in Hyperthermia. II. The Acid-Base Equilibrium in Hyperthermia Induced by Short Radio Waves, J. Biol. Chem. 90: 321, 1931.
9. Cullen, S. C., Weir, E. F., and Cook, E.: Rationale of Oxygen Therapy During Fever Therapy, Anesthesiology 3: 123, 1942.
10. Looney, J. M.: The Relation Between Specific Gravity of Blood Serum and Its Protein Concentration, J. Lab. & Clin. Med. 27: 1463, 1942.

CIRRHOSIS OF THE LIVER: THE LIPOTROPIC ACTION OF PARENTERALLY ADMINISTERED AMINO ACIDS

I. Donald Fagin,* M.D., Melville Sahyun,† Ph.D., and Ralph W. Pagel,* M.D.
Detroit, Mich.

In a recent publication[1] an investigation of the possible therapeutic effects of intravenously administered amino acids‡ in five patients with cirrhosis of the liver was reported. Briefly, the results were (1) subjective improvement of great or moderate degree in four of the five patients during the one month period when the amino acids were used; (2) shrinkage in size of the liver and spleen in all patients in whom these organs were enlarged; (3) disappearance of peripheral edema; (4) indications of improvement in hepatic function, as gauged by hippuric acid synthesis and glucose tolerance tests in all cases, and improvement in bromsulfalein excretion in one case. On the basis of published experimental data it was postulated that the decrease in size of the liver was

*Medical Service and Laboratories, U. S. Marine Hospital, Detroit, Mich.
†Division of Biochemical Research, Frederick Stearns & Company, Detroit, Mich.
‡Fortified acid hydrolysate of casein supplied through the courtesy of Frederick Stearns & Company.

Received for publication, Dec. 14, 1942.

due to the resorption of fat through the lipotropic action of the amino acids. The decrease in splenic size was interpreted as resulting from a decongestion secondary to improved portal circulation.

This report presents data derived from analyses for total lipid and total nitrogen content of the livers of three groups of patients: (1) those who died with cirrhosis of the liver but received amino acid therapy; (2) patients who died with cirrhosis of the liver who did not receive amino acid therapy; and (3) patients who died from other causes without cirrhosis of the liver. The value of this study arises through an opportunity to compare data collected from the chemical study of human livers with those published from similar studies in experimental animals.

Of the five patients who received parenteral amino acids, three (G. R., A. G., and P. W.*) evidenced sufficient clinical improvement to warrant discharge from the hospital. The remaining two patients (J. R. and G. B.*) maintained a clinical plateau for two and one-half and three and one-half months, respectively, following the month of amino acid administration, then entered a rapid and progressive downhill course and died in the hospital. All five patients exhibited a very slight increase in liver size when the administration of amino acids was discontinued; however, in no case did the liver return to its original degree of enlargement, presumably because the patients were maintained on an adequate diet.

To the patients studied and reported, we have added E. C., a patient with chronic alcoholism and advanced "decompensated" cirrhosis of the liver, who died two months after having received amino acids. Autopsies were performed on the patients who died in the hospital (G. B., J. R., and E. C.), and the livers grossly and microscopically exhibited the characteristic pathologic features of portal cirrhosis of the Laënnec or "alcoholic" type. All three patients had a background of chronic alcoholism.

MATERIAL AND METHOD

Formalin-fixed specimens of liver obtained at autopsy from three groups of patients were analyzed for total fat and total nitrogen content; the duration of formalin fixation of the specimens varied from a few days to eighteen months.

Group I consisted of the three patients described above who had cirrhosis of the liver and chronic alcoholism and received parenteral amino acids for three weeks (E. C.) or one month (J. R. and G. B.) during their course.

Group II consisted of patients who had cirrhosis of the liver and chronic alcoholism but had not received amino acids. We reviewed our autopsy protocols for the eighteen-month period prior to this study and found four such cases. No cases with cirrhosis of the liver and chronic alcoholism other than the seven included in Group I and II came to autopsy during this eighteen-month period.

Group III consisted of ten patients who were unselected except that none presented post-mortem evidence of primary liver disease; this group served as a control.

*Abstracts of the clinical records of these patients may be found in Reference 1.

The liver specimens, preserved in 4 per cent formaldehyde, from these three groups of patients were analyzed by the biochemist for total liver fat and total liver nitrogen. These specimens were identified to the biochemist by number only. Glycogen determinations were not done. The analyses for fat were performed, with minor modifications, by the chloroform extraction method used by Channon, Platt, and Smith.[2]

The effect of formalin fixation on liver lipids was investigated by Halliday,[3] who found that, although total solids and total cholesterol content were not significantly affected by treatment with formalin, the total fatty acid content decreased. The decrease was only slight during the first three months of formalin fixation but was considerable after longer periods, probably because of auto-oxidation. Weil,[4] working with nervous tissue, found that phosphatides were hydrolyzed after formalin treatment, but cholesterol and galactolipids were not appreciably changed. In view of these observations, lipoid partition determinations of our fixed specimens were considered inadvisable. We have found that specimens kept in formalin at room temperature show lower fat content than specimens from the same liver kept in the refrigerator, probably because of changes in the properties of certain phospholipids which either tend to diffuse into the formaldehyde or, upon treatment with formaldehyde, become less soluble in fat solvents such as chloroform. It must be emphasized that the analyses made on formalin-fixed liver tissues do not represent accurately the concentrations of fat in the fresh tissues but must be considered merely as reflections of the fresh tissue values. Each fat analysis was done in duplicate, and the differences between the duplicate determinations varied from 0 to 0.14 Gm. per 100 Gm. of tissue, averaging 0.09 Gm. per 100 Gm. of tissue.

RESULTS

The results of the analyses of the livers of the patients in Group I are presented in Table I. The average total fat content of these livers from patients with chronic alcoholism and cirrhosis who had received amino acids was 2.16 Gm. per 100 Gm. of tissue, and the average total liver protein (calculated from the total nitrogen) was 19.0 Gm. per 100 Gm. The liver weight averaged 1,607 Gm.

In Group II, consisting of patients who had had chronic alcoholism and cirrhosis of the liver but had not received amino acids, the total fat content of the liver specimens averaged 22.06 Gm. per 100 Gm. of tissue and the total protein content averaged 13.1 Gm. per 100 Gm. The range for total fat was

TABLE I

PATIENTS WITH CIRRHOSIS OF THE LIVER AND CHRONIC ALCOHOLISM TREATED WITH AMINO ACIDS*

PATIENT	LIVER WEIGHT (GM.)	TOTAL LIVER LIPIDS GM. PER 100 GM. OF TISSUE	TOTAL LIVER NITROGEN GM. PER 100 GM. OF TISSUE	TOTAL LIVER PROTEIN GM. PER 100 GM. OF TISSUE
J. R.	1,400	2.13	2.95	18.4
A. B.	2,360	1.89	3.09	19.3
E. C.	1,060	2.45	3.09	19.3
Average	1,607	2.16	3.04	19.0

*The liver weight was determined at the time of autopsy. The liver lipids and nitrogen were determined on formalin-fixed specimens. The total protein was derived from the total nitrogen determination.

from 2.23 to 35.60 Gm. per 100 Gm. and for total protein, from 3.2 to 19.0 Gm. per 100 Gm. The average liver weight was 2,738 Gm. The individual analyses are presented in Table II. [Ralli and her collaborators,[5,6] analyzing fresh liver specimens, found that the average total lipid content of livers from twenty-five patients with a history of chronic alcoholism (including eight patients with evidences of early or advanced cirrhosis) was 12.2 Gm. per 100 Gm. of tissue, with a range of from 2.0 to 34.8 Gm. per 100 Gm. The average liver weight of their group was 2,020 Gm. The eight patients in her group with cirrhosis of various stages and alcoholic histories had an average total liver lipid of 13.0 Gm. per 100 Gm. (with a range of 2.00 to 34.8 Gm. per 100 Gm.), and an average liver weight of 2,519 Gm.]

TABLE II

PATIENTS WITH CIRRHOSIS OF THE LIVER AND CHRONIC ALCOHOLISM WHO DID NOT RECEIVE AMINO ACIDS

PATIENT	LIVER WEIGHT (GM.)	TOTAL LIVER LIPIDS GM. PER 100 GM. OF TISSUE	TOTAL LIVER NITROGEN GM. PER 100 GM. OF TISSUE	TOTAL LIVER PROTEIN GM. PER 100 GM. OF TISSUE
J. L.	1,450	2.23	3.04	19.0
G. C. B.	3,950	35.60	0.485	3.2
W. L.	3,900	32.98	1.96	12.2
C. E. D.	1,650	17.41	2.90	18.1
Average	2,738	22.06	2.096	13.1

TABLE III

PATIENTS WITHOUT PRIMARY DISEASE OF THE LIVER

PATIENT	DIAGNOSIS	LIVER WEIGHT (GM.)	TOTAL LIVER LIPIDS GM. PER 100 GM. OF TISSUE	TOTAL LIVER NITROGEN GM. PER 100 GM. OF TISSUE	TOTAL LIVER PROTEIN GM. PER 100 GM. OF TISSUE
E. I.	Hypertensive heart disease	2,500	4.33	2.52	15.7
F. A. P.	Prostatic hypertrophy and pyelitis	2,050	1.97		
A. G. D.	Hypertensive heart disease, pulmonary infarction	2,000	1.70		
F. B.	Aortic stenosis	1,700	3.68		
R. D.	Nephrosclerosis	2,000	3.11		
J. L. B.	Pulmonary tuberculosis	1,000	2.07	3.22	20.1
C. C.	Nephrosclerosis	2,100	3.19		
C. C.	Lung abscess	1,300	2.34		
W. V.	Carcinoma of colon	1,650	3.67		
T.	Myocardial infarction	2,250	4.03	3.34	20.8
Average		1,855	3.01	3.03	18.8

In Group III, consisting of patients dying of causes not related to primary disease of the liver, the total fat content of the liver specimens averaged 3.01 Gm. per 100 Gm., with a range of 1.70 to 4.33 Gm. per 100 Gm. The total protein (determined in only three instances) averaged 18.8 Gm. per 100 Gm. The liver weight averaged 1,855 Gm. [Ralli and co-workers[5] found that fresh livers from normal individuals who died of accidental injuries averaged a total lipid content of 4.98 Gm. per 100 Gm. of liver (with a range of 2.42 to 8.41 Gm.) and the liver weight averaged, 1,430 Gm.] The rather high average

liver weight of our patients in this group is due to the fact that in several patients chronic passive congestion of the liver secondary to circulatory insufficiency was present. The individual analyses are presented in Table III.

Examination of slides prepared from the livers analyzed revealed a rather close correlation between the extent of fatty infiltration evident microscopically and the amount of fat determined by analysis.

DISCUSSION

Admittedly, the number of cases is small and of little statistical significance. However, available data present very few studies of this character. Our comments here will concern themselves wholly with the type of cirrhosis described by Connor as "fatty cirrhosis."[7] The patients in Groups I and II are examples of this type of cirrhosis, as distinguished from toxic, biliary, syphilitic, and pigment cirrhosis.

From the data presented, two inferences are permissible:

(1) The percentage of liver protein in general varies inversely with the percentage of liver fat. Any discrepancies may be accounted for by changes in the glycogen content. It must be remembered that at least a part of the apparent increase in hepatic protein may be relative rather than actual, since the decrease in fat content would of itself result in an increased percentage of protein. The protective action of protein stores in the liver and body against hepatotoxic agents has been demonstrated in the experimental animal by Miller and Whipple,[8] and our analyses are suggestive confirmation of the same mechanism in man.

(2) The administration of the amino acid mixture to patients with cirrhosis of the liver and chronic alcoholism apparently resulted in a decrease in percentage of hepatic fat and a concomitant increase in percentage of hepatic protein. Of course, the ideal method of proof that the changes in liver fat and protein are a direct result of the action of the amino acids would be the determination of fat and protein content of liver biopsy specimens before and after the administration of amino acids; however, this procedure was not feasible in the patients we studied. The fact that all the patients in Groups I and II were chronically addicted to alcohol renders them a fairly homogeneous group and reduces the number of variable factors, as also does the determination of average values. It will be seen that, in Group II, the liver of only one patient (J. L.) exhibited fat and protein percentages and liver weight within the control range and within the range of specimens from patients treated with amino acids. Connor[7] states that in patients who have used alcohol excessively over a long period of time but have maintained a fairly good diet, fat may or may not be present and the liver is likely to be reduced in size.

The significance of fatty infiltration of the liver as a precursor of cirrhosis has been stressed recently.[7, 9] In experimental animals, fatty livers have been produced by high fat diets, cystine, starvation, cholesterol, and toxic agents such as alcohol, phlorizin, chloroform, carbon tetrachloride, phenylhydrazine, and phosphorus.[10, 11] In clinical medicine, fatty livers may develop in chronic alcoholism, diabetes, mellitus, pernicious anemia, poisonings with the chemicals mentioned above, yellow fever, eclampsia, acute yellow atrophy, marked obesity,

certain endocrine disorders, and lesions of the pituitary or midbrain. Fatty infiltration of the liver may lead to interference with tissue metabolism, with resultant degeneration of liver cells and replacement by perilobular fibrous tissue. It is readily seen that any agent which would decrease the rate of deposition of liver fat and accelerate its removal (i.e., a lipotropic agent[12]) might prevent the development of cirrhosis in fatty livers and would be of vast clinical significance. Recent theories of the pathogenesis of hepatic cirrhosis have been reviewed by Jolliffe and Jellinek,[13] and they express the belief that the investigations of Connor and his associates in the relationship between fatty liver and cirrhosis are among the most important advances.

The experimental background of lipotropic activity of raw pancreas, lecithin, choline, proteins, methionine, betaine, and constituents of vitamin B complex has recently been reviewed.[12] It has been shown that choline, methionine, and betaine are similar physiologically in their lipotropic activity and are similar chemically in that all possess labile methyl groups.[14] The lability of the methyl group and its potentiality for participating in transmethylation reactions may be the determining factor in the lipotropic activity of these substances and may be important in detoxification processes in the body. Lowry and co-workers[15] reported that the administration of choline or a high casein diet to rats with experimental dietary cirrhosis resulted in regeneration of liver cells, disappearance of degenerative fatty changes, and decrease in liver size. Daft, Sebrell, and Lillie[16] demonstrated that cirrhosis of the liver could be *prevented* by the administration of methionine or choline or casein, singly or in combination, to rats receiving low protein rations and alcohol. The methionine content of casein is 3 per cent,[17] and it has been shown that methionine may supply methyl groups, for the formation of choline or an active compound derived from choline,[18] and that the administration of methionine accelerates the rate of phospholipid turnover in the liver.[19] Recognizing that results in experimental animals are not directly applicable to the interpretation of metabolic changes in human beings, we believe, nevertheless, that the lipotropic activity of the amino acids administered in our study probably was due to the methionine content of the amino acid mixture, which was prepared by hydrolyzing casein, and contains amino acids in the same proportion as they are found in casein. The lipotropic activity of choline has been demonstrated conclusively,[11, 12, 15, 20] and the good results reported by Broun and Muether,[21] in treating patients with hepatic cirrhosis with choline and a low fat diet, may, in part, be due to the lipotropism of choline.

Good results have been reported in treating cirrhosis of the liver with a high carbohydrate, high protein, low fat diet, with vitamin supplements,[22, 23] and it remains to be seen whether the prognosis can be influenced *still* more favorably by the additional use of choline, methionine, or amino acid mixtures.

SUMMARY

1. Liver specimens (preserved in formalin) from patients with cirrhosis of the liver and chronic alcoholism were analyzed for their total lipid and total nitrogen content. It was found that specimens from patients who had received amino acids as part of their therapy contained a greater percentage of protein

and a lesser percentage of fat than specimens from patients who had not received amino acids.

2. It is postulated that these results indicate a lipotropic activity of the amino acid mixture, probably due primarily to their methionine content.

3. The percentages of liver fat and of liver protein were found to vary inversely.

4. The clinical significance of these findings is briefly discussed.

REFERENCES

1. Fagin, I. D., and Zinn, F. T.: Cirrhosis of the Liver: Results of Treatment with Parenterally Administered Amino Acids, J. LAB. & CLIN. MED. 27: 1400, 1942.
2. Channon, H. J., Platt, A. P., and Smith, A. R.: The Dietary Prevention of Fatty Livers, Biochem. J. 31: 1736, 1937.
3. Halliday, N.: The Effect of Formalin Fixation on Liver Lipids, J. Biol. Chem. 129: 65, 1939.
4. Weil, A.: The Influence of Formalin Fixation on the Lipoids of the Central Nervous System, J. Biol. Chem. 83: 601, 1929.
5. Ralli, E. P., Rubin, S. H., and Rinzler, S.: The Liver Lipids in Normal Human Livers and in Cases of Cirrhosis and Fatty Infiltration of the Liver, J. Clin. Investigation, 20: 93, 1941.
6. Ralli, E. P., Paley, K., and Rubin, S. H.: The Liver Lipids and Their Distribution in Disease, An Analysis of 65 Human Livers, J. Clin. Investigation 20: 413, 1941.
7. Connor, C. L.: The Etiology and Pathogenesis of Alcoholic Cirrhosis of the Liver, J. A. M. A. 112: 387, 1939.
8. Miller, L. L., and Whipple, G. H.: Chloroform Liver Injury Increases as Protein Stores Decrease, Am. J. M. Sc. 199: 204, 1940.
9. Connor, C. L.: Fatty Infiltration of the Liver and the Development of Cirrhosis in Diabetes and Chronic Alcoholism, Am. J. Path. 14: 347, 1938.
10. Bollman, J. L.: Experimental Studies on Hepatic Alterations (Fatty Liver), Proc. Staff Meet., Mayo Clin. 11: 727, 1936.
11. Best, C. H.: The Role of the Liver in the Metabolism of Carbohydrate and Fat. III. The Deposition of Liver Fat, Lancet 1: 1274, 1934.
12. Frame, E. G.: Lipotropic Substances, Yale J. Biol. & Med. 14: 229, 1942.
13. Jolliffe, N., and Jellinek, E. M.: Vitamin Deficiencies and Liver Cirrhosis in Alcoholism. Part VII. Cirrhosis of the Liver, Quart. J. Studies on Alcohol 2: 544, 1941.
14. Editorial: Significance of the Labile Methyl Group, J. A. M. A. 119: 1111, 1942.
15. Lowry, J. V., Daft, F. S., Sebrell, W. H., Ashburn, L. L., and Lillie, R. D.: Treatment of Dietary Liver Cirrhosis in Rats with Choline and Casein, Pub. Health Rep. 56: 2216, 1941.
16. Daft, F. S., Sebrell, W. H., and Lillie, R. D.: Production and Apparent Prevention of a Dietary Liver Cirrhosis in Rats, Proc. Soc. Exper. Biol. & Med. 48: 228, 1941.
17. Block, R. J., and Bolling, D.: The Determination of the Amino Acids, Minneapolis, 1940, Burgess Publishing Co.
18. Channon, H. J., Manifold, M. C., and Platt, A. P.: The Action of Sulfur-Containing Amino Acids and Proteins on Liver Fat Deposition, Biochem. J. 34: 866, 1940.
19. Pearlman, I., Stillman, N., and Chaikoff, I. L.: Radioactive Phosphorus as an Indicator of Phospholipid Metabolism, J. Biol. Chem. 133: 651, 1940.
20. György, P., and Goldblatt, H.: Experimental Production of Dietary Injury (Necrosis, Cirrhosis) in Rats, Proc. Soc. Exper. Biol. & Med. 46: 492, 1941.
21. Broun, G. O., and Muether, R. O.: Treatment of Hepatic Cirrhosis with Choline Chloride and Diet Low in Fat and Cholesterol, J. A. M. A. 118: 1403, 1942.
22. Patek, A. J., Jr., and Post, J.: Treatment of Cirrhosis of the Liver by a Nutritious Diet and Supplements Rich in Vitamin B Complex, J. Clin. Investigation 20: 481, 1941.
23. Fleming, R. G., and Snell, A. M.: Portal Cirrhosis With Ascites: An Analysis of 200 Cases with Special Reference to Prognosis and Treatment, Am. J. Digest. Dis. 9: 115, 1942.

LABORATORY METHODS

GENERAL

A SIMPLE METHOD FOR THE SEPARATION OF BLOOD SERUM*

Lea C. Steeves, B.A., M.D., Montreal, Can.

THE following method has been found to be simple and timesaving in the preparation of arsphenaminized serum for intraspinal therapy. The principle is adapted from commercial plasma separation and transfusion sets and can be used for several types of cell serum or cell plasma separation.

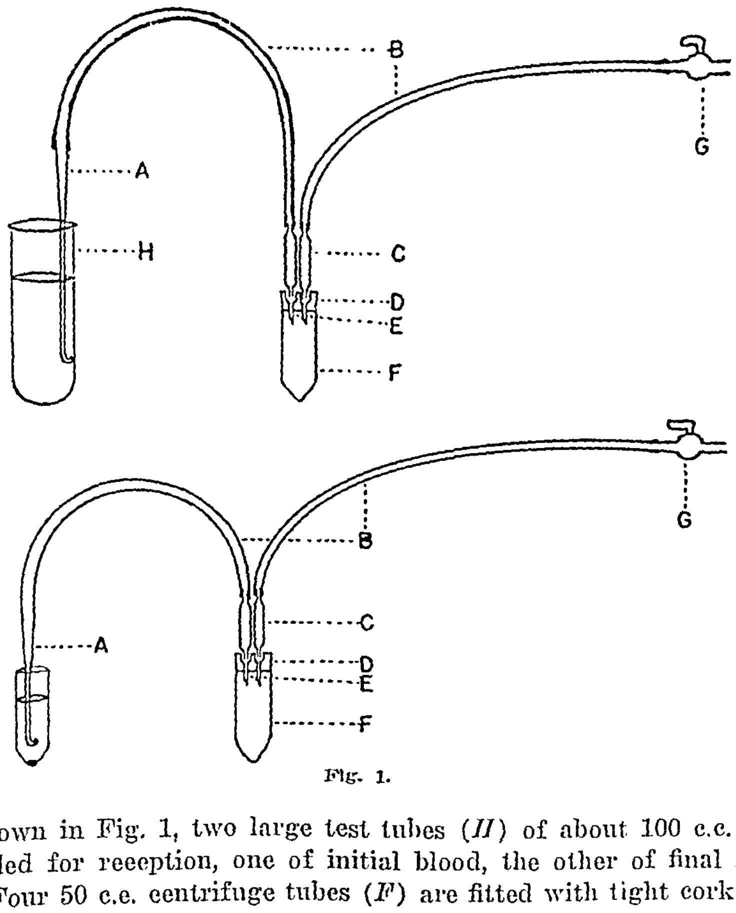


Fig. 1.

As shown in Fig. 1, two large test tubes (*H*) of about 100 c.c. capacity are provided for reception, one of initial blood, the other of final serum or plasma. Four 50 c.c. centrifuge tubes (*F*) are fitted with tight cork stoppers

*From the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal.

Received for publication, July 2, 1942.

Miss Doris Brophy suggested the pipettes and prepared Fig. 1.

(*D*),* through each of which two 18-gauge blood needles (*E*) are inserted. The hubs of the needles are countersunk in the cork, and one-half an inch is cut off the top of the centrifuge tube in order that the assembly may spin freely in the ordinary laboratory centrifuge. A glass adaptor (*C*), as commonly used in intravenous sets, is provided for each needle, and to each adaptor a short length of rubber tubing (*B*) is attached. A glass pipette (*A*) with approximately 18-gauge bore at the U tip is provided for each centrifuge tube. Laboratory vacuum taps (*G*) provide the necessary suction.

The entire apparatus can be neatly packed for sterilizing, wrapped under one cover, in the following units: (1) initial and final reception tubes; (2) centrifuge tubes with corks and needles inserted; (3) adaptors, tubing, and pipettes; (4) adaptors and tubing for connection to unsterile vacuum tap.

Assembly can be carried out by one ungloved operator without contamination. Serum or plasma can be separated with very little waste because of the U pipette which reduces upward currents from the cell fluid interface. By removing the adaptors, unit two can be centrifuged uncontaminated to remove residual blood cells. This procedure can be repeated as desired with loss of only a very few cubic centimeters of fluid upon each pipetting. In the case of arsphenaminized serum, Fordyce, as quoted by Moore,[1] centrifuges twice.

SUMMARY

A simple, semiclosed apparatus for rapid separation of cell serum or cell plasma preparations is described. It can be readily assembled from laboratory materials and can be used by one person unaided without danger of contamination.

REFERENCE

1. Moore, J. E.: The Modern Treatment of Syphilis, Baltimore, 1933, Charles C Thomas.

A QUADRUPLE STAINING METHOD FOR TISSUES†

S. S. KALTER, LAWRENCE, KAN.

RECENT advances in staining procedures show a definite trend toward the employment of various differential staining methods for the study of normal as well as pathologic tissues. The evolution of staining techniques need not be discussed here, as the interested student need only to turn to a monograph by Conn[1] for further information.

The foundation for this quadruple stain was Flemming's triple stain (1891), which appears to be the basis for various other multiple staining methods. It seems, however, for obvious reasons, that histologists and pathologists, in the

*Tapered rubber stoppers give a more air-tight fit but must be renewed after fifteen to twenty autoclavings.

†From the Department of Bacteriology, University of Kansas.
Received for publication, July 7, 1942.

main, restrict their tissue staining to hematoxylin-eosin, with Mallory's[2] connective tissue stain being employed when a tinctorial differentiation was wanted. The botanists, on the other hand, basing their procedures on Flemming's stain, as did Conant[3] and Johansen,[4] investigated the applicability of more complex procedures for the study of mitosis as well as differentiation of botanical tissues.

Dratman,[5] following Conant's work, proceeded to introduce this technique for the study of animal tissue. The work herein described is, therefore, a continuation of the work of Dratman. This technique is essentially that of Dratman, with modifications made to insure more constant results and to bring out sharper differentiation of the cellular elements.

METHOD

Tissues are fixed and dehydrated by any preferred method. We have given preference to Bouin's fixative, as it was demonstrated by Baird,[6] to produce a minimum amount of shrinkage and distortion in the cellular elements, with uniform results and delicacy of fixation. In using Bouin's solution, the picric acid must be removed by using a few drops of lithium carbonate in the 70 per cent alcohol dehydration series. In the use of Zenker's fixative the mercuric chloride must be removed.

The tissues, after being embedded in paraffin, give the best staining results when sectioned at 7.5 micra. In affixing the tissues to slides, the loss of tissues is negligible if the slides are thoroughly dried in an incubator at 37° C. overnight. The use of albumin water causes a cloudiness to appear in the staining reaction.

Of utmost importance is the preparation of the dyes. The dye solutions prepared as follows, have been found to give the best results:

1. Safranin O:* 0.2 Gm. of safranin O in 100 c.c. of 50 per cent ethyl alcohol plus 4 c.c. of formalin to act as a mordant and 0.5 Gm. of sodium acetate to accentuate the color.

2. Gentian violet (crystal violet):† 0.5 Gm. in 100 c.c. water.

3. Fast green FCF:‡ A saturated clove oil solution is made using 0.5 Gm. of the dye in 80 c.c. of the oil. The dye is left in the clove oil twenty-four hours with occasional shaking, then filtered through cotton. To 40 c.c. of this solution is added 30 c.c. of orange II.

4. Orange II:§ Saturate clove oil with 0.5 Gm. of the dye and allow to stand twenty-four hours with occasional shaking and filter through cotton.

The staining procedure is as follows: (1) Slides run down to tap water; (2) stained in safranin O, 24 hours; (3) tap water wash; (4) gentian violet, 1 to 2 minutes; (5) tap water wash; (6) 50 per cent ethyl alcohol, 2 minutes; (7) 95 per cent ethyl alcohol, 2 minutes; (8) fast green-orange II—differentiate under the microscope after 5 minutes; (9) pure clove oil for 10 minutes; (10) pure orange II—differentiate under the microscope after 15 minutes; (11) xylol (two changes), 10 minutes each.

*Henry Heil Chemical Co., US-8; National Aniline and Chemical Co., Certification No. NS-8.

†Gruber 9.37; National Aniline and Chemical Co., Certification No. NC-18.

‡National Aniline and Chemical Co., Certification No. NGF-4.

§Coleman and Bell Co.

In steps 8 and 10 the time expressed is a minimum due to various factors such as thickness of the tissues and cellular activity. The tissues are differentiated under the microscope in step 8 and are removed as soon as the connective tissue is green. Further differentiation takes place in the pure clove oil as well as in the orange II; therefore, clearing in the pure clove oil for ten minutes is essential.

RESULTS

Nuclei stain red, with the nucleoli staining purple or a bright purple-red, while the nuclear membrane stains a dark red. The cellular cytoplasm stains from pink to red, with a marked exception being noted in the medulla of the kidney. Here the cytoplasm of the cells making up the descending and ascending limbs of Henle's loop stain light green. The connective tissue stains green, while large elastic fibers or areas where elastic tissue predominates are sharply differentiated in yellow. The fibroblasts are green with purple nuclei. Muscular tissue usually stains a light tan, with the striations as dark bands. Red blood cells are orange. The polymorphonuclears have purple nuclei.

DISCUSSION

This method, although it is a differential connective tissue stain, cannot be compared with Mallory's. The time element as well as the striking connective tissue differentiation obtained by Mallory's stain makes it one of the most outstanding differential techniques now employed.

On the other hand, the main feature obtained by this quadruple stain is the benefits obtained by students of histology. Because of the lightness of the colors, the various elements of the tissues are not masked. The tissues can then be studied from the structural aspects as well as by the differential staining.

REFERENCES

1. Conn, H. J.: History of Staining, Geneva, N. Y., 1933, Commission on Standardization of Biological Stains.
2. Mallory, F. B.: A Contribution to Staining Methods, J. Exper. Med. 5: 15, 1900.
3. Conant, G. H.: Conant's "Triarch" Quadruple Stain, Histological Technic, Krajian, A. A., St. Louis, 1940, The C. V. Mosby Company.
4. Johansen, D. A.: A Quadruple Stain Combination for Plant Tissue, Stain Tech. 14: 125, 1939.
5. Dratman, M. L.: A Quadruple Stain for Animal Tissue, B.A. Thesis, LaSalle College, Philadelphia, Pa., 1940.
6. Baird, T. T.: Comparative Study of Dehydration, Stain Tech. 13: 9, 1938.

SURFACE GROWTH OF CLOSTRIDIUM WELCHII

EDWARD STEINFIELD, M.D., AND MARGARET WATSON, PHILADELPHIA, PA.

DUE to the recent investigations of Kligler and Guggenheim,[1] it has been demonstrated that the determining factor in anaerobiosis is the reduction potential of the medium rather than other factors. In their experiments they found that growth of *Clostridium welchii* occurred in tubes with over 95 per cent of normal oxygen tension, provided an adequate amount of ascorbic acid was present. The reduction potential at which growth occurred was about −0.125 volt. These observations supplemented earlier reports of Lepper and Martin[2] who found by potentiometer and indicator methods that the Eh of freshly prepared cooked meat mediums was of the order of −0.174 volt to −0.2 volt, 1 or 2 cm. from the surface. Since cooked meat medium with a petrolatum seal is uniformly satisfactory in the cultivation of *Cl. welchii* and similar anaerobes, it is obvious that the reduction potential can be taken as a measure of the availability of an anaerobic method regardless of the hypothetical explanation of its mechanism. We have had occasion to verify the statements of Lepper and Martin and have obtained similar readings of the Eh of sealed cooked meat medium tubes by indicator methods.

The practical implication of the work of Kligler and Guggenheim is in the possibility of surface growth of *Cl. welchii* and similar anaerobes which are important, particularly in gas gangrene. Among the various methods which make this possible are the Spray anaerobic dish and the Brewer anaerobic jar. The latter is particularly valuable in the cultivation of obligate anaerobes. The possibility of using stock equipment and material has led us to improvise upon the observations of Kligler and Guggenheim with the use of the ordinary Petri dish.

The method is as follows:

Test tubes containing 12 to 15 c.c. of plain agar are heated to melting point and allowed to cool slowly as in the usual preparation for blood agar plates. Sterile defibrinated blood* is added and immediately thereafter 50 mg. of the sodium salt of ascorbic acid. The latter can be obtained commercially in the form of one cubic centimeter ampules, each containing 100 mg. of sodium ascorbate in solution. These may be diluted with equal volumes of normal saline solution and the appropriate amount added to the blood agar tube. The treated blood agar is poured into sterile Petri dishes and allowed to solidify. Within one-half to one hour the plate darkens due to the reduction of hemoglobin. Circular seals of cellophane slightly smaller than the inner compartment of the Petri dish are cut out, immersed in 70 per cent alcohol for several minutes, and

From the Laboratory of the Jewish Hospital, aided by a grant from the Sidney A. Aloe Research Fund.

Received for publication, July 7, 1942.

*Horse blood is preferable in demonstrating clear-cut beta hemolysis by *Cl. welchii*, but sheep or human blood may be used for convenience.

dried between layers of sterile filter paper. They may be kept in several such layers within sterile Petri dishes. Autoclave sterilization is less desirable since it tends to wrinkle the cellophane. We have found the product commercially labeled "No. 300 moisture proof" quite satisfactory. After streaking cultures or material presumably containing *Cl. welchii* over the surface of the treated blood agar, a cellophane seal handled with a flamed forceps is gently placed over the agar surface and rolled smoothly against it with a bit of sterile glass tubing threaded upon a wire. By this means the oxygen tension is kept to a minimum; the plate can be examined by transmitted light and handled in any position. Subcultures can easily be made by lifting the edge of the cellophane cover with forceps without undue distortion of the surface. Hemolysis can be judged, though not quite so sharply as in the usual blood agar plate because of the reduced hemoglobin. It is quite possible to obtain somewhat similar results with stored blood agar plates by applying the same amounts of sodium ascorbate to the surface of the plate and carrying the material along with the platinum loop or sterile swab during streaking. In such preparations it is possible to note the areas where the reducing action is lacking with consequent absence of growth. The method in general has so far been applied to strains growing under laboratory conditions. However, since these cultures fail to grow by ordinary aerobic methods, it can be assumed that their anaerobic properties are still maintained. In order to verify the sealing effect of the cellophane, agar plates containing the sodium ascorbate and an indicator such as 2, 6-dibromophenolindophenol were set up and kept at room temperature. All plates showed decolorization of the dye, but controls without cellophane gradually exhibited oxidation from the surface downward; those with cellophane remained decolorized for from seven to ten days. Although this indicator does not register the optimum Eh which can be obtained, it is useful in this particular observation because of its clearcut readings.

There are certain disadvantages to the method. So far it has been found difficult to apply to the sugar mediums and for the culture of *Cl. tetani.* A moisture film may make single colony isolation difficult. The expenditure for the sodium ascorbate would have to be considered in large scale observations. It may be advisable to adapt this method, if possible, to the use of such reducing mediums as cysteine hydrochloride or sodium thioglycollate. These have recently been reviewed and amplified by Brewer.[3] Spray[4] has also mentioned the use of flat sheets of cellulose over deep agar plates. Although these approaches to surface culture are no doubt being investigated, we feel that this brief description may induce trial and improvement of such methods. A similar technique may prove useful in some exigencies of time and material or where the use of hydrogen jars is undesirable.

REFERENCES

1. Kligler, I. J., and Guggenheim, K.: Influence of Vitamin C on Growth of Anaerobes in the Presence of Air, J. Bact. 35: 141, 1938.
2. Lepper, E., and Martin, C. J.: Oxidation-Reduction Potential of Cooked Meat Media, Brit. J. Exper. Path. 11: 137, 1930.
3. Brewer, J. H.: Clear Liquid Mediums for the "Aerobic" Cultivation of Anaerobes, J. A. M. A. 115: 598, 1940.
4. Spray, R. S.: Composition Tubes as Substitute for Glass in Isolation of Sporulating Anaerobes, J. Lab. & Clin. Med. 26: 1957, 1941.

ADAPTATION OF A KAHN SHAKER FOR HOMOGENIZING SPECIMENS FOR BACTERIOLOGIC PROCEDURES

Lillian Buxbaum,* M.S., New York, N. Y.

In the course of our laboratory work it is often necessary to shake vigorously several different types of bacterial samples to obtain homogenous suspensions. To expedite and simplify this procedure, a mechanical shaker is considered desirable. A Kahn shaker that was already part of our laboratory equipment was satisfactorily adapted for our needs. The idea for adapting a Kahn shaker was obtained from a bottle shaker manufactured by the Precision Scientific Company of Chicago (catalogue No. 5892).

Fig. 1.

Lead-coated copper, which is rust-resistant, 1 mm. in thickness, was used to make a tray 14 inches long, 12 inches wide, and 2 inches deep (Fig. 1). Specifications of the tray were limited by the apparatus already in the shaker which is used for shaking hemocytometers. Both the tray and the latter apparatus are fitted into the ledge of the shaker. The tray is removable so that it can be easily cleaned and sterilized.

Metal holders (Fig. 2) were ordered from the Standard Scientific Supply Company of New York. Spring brass was the metal of choice for these holders because of its malleability and tensile strength. Two 1 mm. metal bands, 1 inch wide and 6 inches long, are placed at right angles and soldered together. The bands are bent at a 90-degree angle so that the four upright arms are 2 inches in height. The four ends are curved in order to hold the specimen containers

Received for publication, July 14, 1942.

*Assistant Bacteriologist, Pathological Laboratory, Willard Parker Hospital.

securely. The width at the base of the finished holder is 2 inches. The top can be expanded to 4 inches or contracted to 2 inches, depending on the size of the container used. Rivets are used to attach the holders to the tray at a distance of three and one-half inches from the center of each holder.

The shaking apparatus, thus modified, was utilized at first to homogenize sputum for concentration of *Mycobacterium tuberculosis.* Half pint wax cups with a 2-inch base diameter, a 3-inch brim diameter, and 3½ inches in height were used as containers for these specimens. All tenacious masses were completely broken up, and good digestion of the sputum was obtained after adding 3 per cent sodium hydroxide and shaking for twenty minutes.

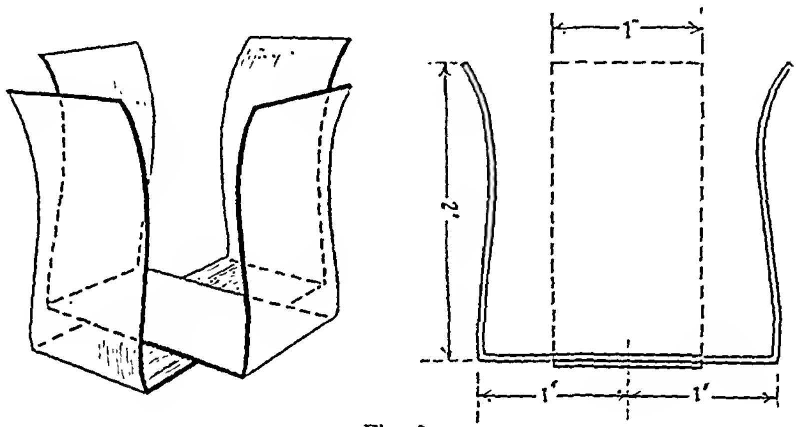


Fig. 2.

Due to the fact that the holders can be adjusted, different sizes and types of containers can be inserted and held securely by the flexible metal strips. Among other uses, it was found that homogenous suspensions of streptococcus growth in some liquid media resulted after shaking for from four to six minutes. As this organism produces a granular growth in broth, the shaker proved invaluable when used in preparing different amounts of antigens for the Griffiths' typing of hemolytic streptococci.

The hospital tinsmith, Frank J. Musacchia, Jr., constructed the tray and riveted the holders.

I wish to thank Dr. Vera B. Dolgopol for her suggestions which aided in the preparation of this paper.

ROLLER RIM-DRIVE KYMOGRAPHS

R. P. WALTON, M.D., F. M. COOK, B.S., AND A. B. CULLEN, M.S.,
CHARLESTON, S. C., AND UNIVERSITY, MISS.*

KYMOGRAPHS have always represented one of the major equipment problems of pharmacology and physiology laboratories. The chief difficulty in their construction is occasioned by the need for wide ranges of speed, and, at present, these are obtained almost exclusively by shifting gears or rearranging belts, with usually a rheostat or governor control of the motor. These motor controls are limited to a 6-to-1 range usually, or as a maximum, a 10-to-1 range. Additionally, the rheostat control even at the 6-to-1 range involves serious loss of torque at the lower speeds, since its effect is essentially to dissipate power. This, similarly, is a disadvantage of fan-controlled spring motors.

A distinct improvement will be the development of some device which will give smooth, continuous control of small motors over a speed range of about 100 to 1. By the use of reducing gears, two such speed areas could be made available with only one shift of gears (or change of drives). Speed ranges of 10,000 to 1 would then be available, and this covers practically all the needs of pharmacologic and physiologic work. A wide range of control is now commercially available with large motors. For instance, the Crocker-Wheeler polyspeed motors operate with a variable speed 3 phase A. C. motor which has separately excited rotor and stator coils and has essentially the same characteristics as a D. C. shunt motor; that is, constant torque at different speeds.[1] Another possible type of motor control is that described by Ryder in which a D. C. motor is shunt wound and has a separately excited armature and field.[2] The armature voltage might be varied from zero to maximum by means of a grid-controlled rectifier acting as a variable voltage source, a function which can be satisfactorily performed by a thyratron tube. There are other arrangements of a purely mechanical nature by which the desired motor control might be obtained. One is by the use of small hydraulic, piston-drive motors and pumps (in which variations of fluid flow are subject to valve control). Another is by the use of a planetary drive system in which the differential rate of two motors might be subject to a wider range of control than is the case with a single motor.

We have made tentative experiments with each of these possible arrangements and in each case have found enough complications and disadvantages to preclude the immediate prospect of using them as kymograph drives which could be developed as a practical improvement over existing methods. These possibilities are mentioned, however, since with subsequent technical developments or in other hands they might be satisfactorily adapted to this particular purpose.

The arrangement which we have developed and put into use involves a simple mechanical principle which without any new meshing of gears gives the needed wide range of speeds and is capable of construction at a relatively low

*From the Departments of Pharmacology, Medical College of the State of South Carolina and School of Medicine, University of Mississippi.
Received for publication, July 17, 1942.

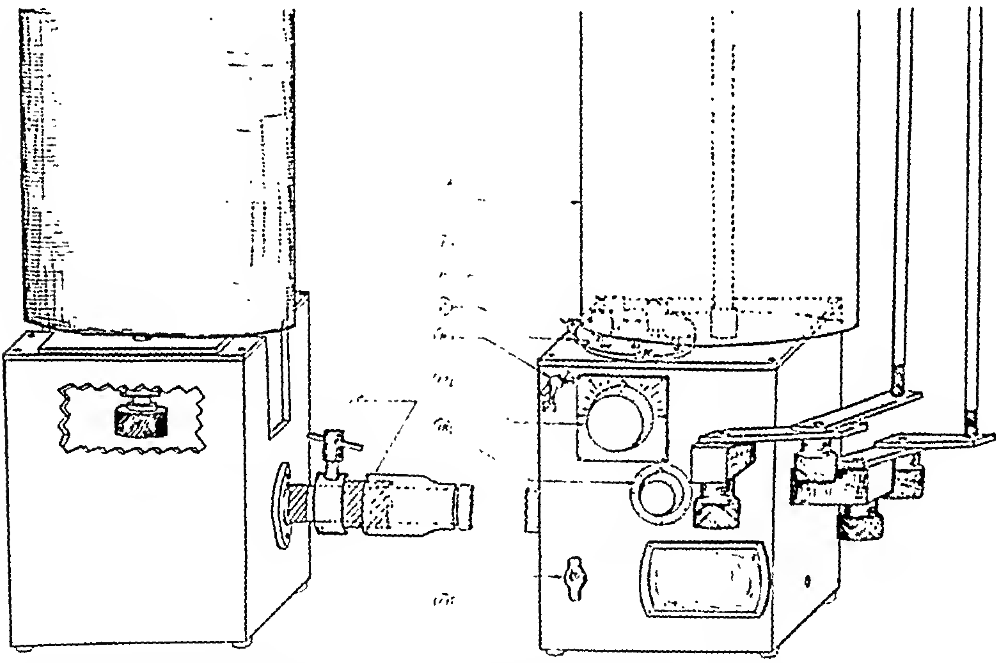
cost under ordinary shop conditions. Its chief point of novelty lies in the use of the roller rim-drive principle such as has been used in musical record players for the last several years. This is essentially a small, power-driven roller operating by friction against the inner shell of a kymograph drum. Commercial friction drives at a more central point of power have not been satisfactory in our experience, but this type of friction drive at the most peripheral point of the system has proved exceptionally satisfactory. One such drive has been used now for five years in these laboratories and has always given smooth operation without slipping. Our presently preferred kymograph design operates at top speed with a pull of ten pounds at the periphery of an eight-inch drum, and, under test conditions up to this maximum pull, there has been no slipping. This is an extreme test, since some widely used commercial kymographs are stopped by less than one-pound pull at the drum periphery.

The unique advantage of the roller rim-drive lies in the fact that several rollers can be power driven within the shell of the drum, and, by a fairly simple arrangement, any single roller can be selected for driving contact while the other rollers are allowed to idle. Thus different ranges of speed can be made immediately available without the usual changing of gears or shifting of drive belts. The fact that there is actually no new meshing of gears in any of these changes of speeds eliminates the need for the more precise type of machining construction which is necessary when several gear shifts are required.

This principle can be illustrated best by the simplest theoretical arrangement in which only two rollers are being driven inside the drum shell. These are being driven at different rates of speed by a motor and gear box in a housing below the drum. The rollers are mounted on free, movable arms of bar iron and connected by small spur gears to the power shafts. The arms rotate freely about cylinders which enclose the fixed power shafts. By two simple maneuvers, one roller can be released from contact with the inside shell of the drum and the other brought into contact. This contact can be maintained with a tension spring. With rheostat control of the motor, which usually gives satisfactory control over a range of 6 to 1, two areas of speed range are made available. This represents the simplest arrangement of multiple drives, and the construction details are simple. By increasing the number of power-driven shafts and their attached rotating arms and roller drives, additional speed ranges are made available and without the necessity of meshing new gear combinations. The mechanical difficulties of arranging side arms underneath the drum, however, increase with the number of side arms and a point may be reached where there may be interference with recording instruments.

We have found that the most suitable means of making available several such speed areas is a system which drives a number of contact rollers from one circular gear box, which itself can be freely rotated about a central power shaft coming directly from the motor. The whole gear housing can be shifted away from the drum periphery enough to release the friction contact of any of its rollers, the gear system rotated to place the selected roller drive in position for contact with the rim, and the gear housing shifted back to establish contact of the selected roller with the rim.

This gear box mechanism is of a clock-work type, and we have preferred a gear reduction of about 6 to 1 between each roller drive shaft. With five such roller drives and ordinary rheostat control, a continuous selection of speeds is available over a range of about 7,500 to 1. This permits a top speed which completely revolves a ten-foot paper in about one minute and a lowest speed which revolves the paper once in about five days. Stated differently, the paper can be driven at about 5.0 cm. per second at top speed and at about 2.4 cm. per hour at the lowest speed. Any speed within this range can be selected immediately and put in operation without disturbing the continuity of the tracing. There is no disturbing jar of the kymograph with speed changes, there is no waiting interval for a train of gears to catch up, and there is no forward jerk of the drum as new gears are meshed. These speed changes are obtained by knob controls placed out of the area of the support rods and recording instruments.



Figs. 1, 4, and 5.—*1*, Drive roller; *2*, indicator plate; *3*, gear housing; *4*, bevel gears; *5*, support for shaft to bevel gear; *6*, coupling; *7*, flexible shafting; *8*, motor mounting; *9*, drum shaft; *10*, stabilizing post; *11*, cam acting on gear housing to disengage roller; *12*, spring acting in gear housing to maintain friction of roller; *13*, rheostat; *14*, motor; *15*, drum; *16*, knob to cam shaft; *17*, knob to bevel gear; *18*, knob to rheostat; *19*, on-off power switch; *20*, spreader arrangement.

Fig. 1.—Schematic view of entire kymograph.

The details of this construction are given below. Additionally some features of kymograph assembly are mentioned which may be helpful to those particularly interested in cutting down costs. A rugged kymograph giving the above speed range and operating with adjustable large double drums can be assembled from less than $35 worth of parts in a shop equipped with a lathe and ordinary metal working equipment.

Drums.—The drums are made of laminated bakelite tubing (Synthane) which can be obtained in varying diameters and can be cut to order in various lengths. We have used such drums for five years and consider them preferable to metal drums. Heads are machined from a plastic slab or, adequately enough,

from plywood with a top covering of smooth asbestos composition board.* The lower head is fixed in the drum about one to two inches from the lower edge. This allows room for the rubber rollers to project up inside the shell of the drum. The drum itself with a central shaft of metal tubing idles on a shaft of three-fourth-inch diameter rodding attached to the base.

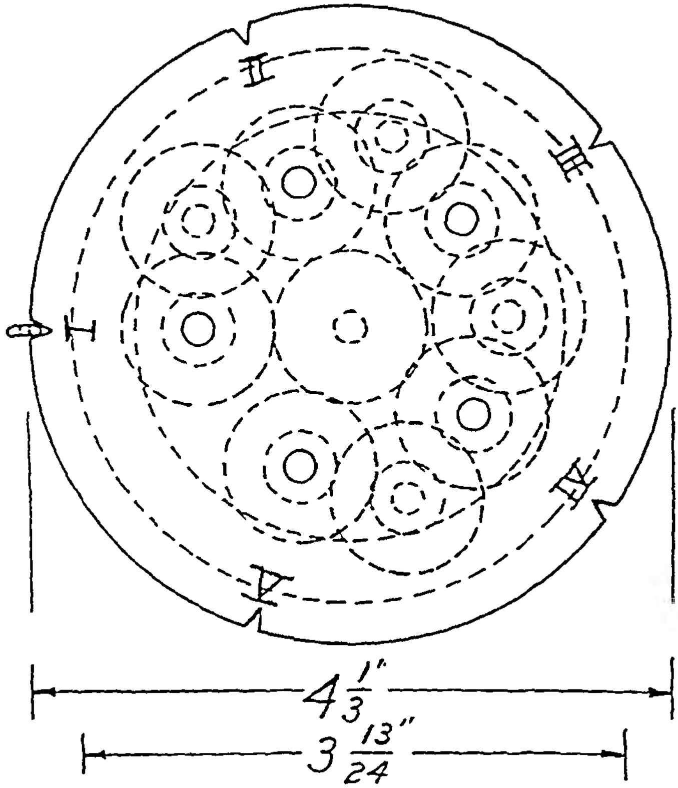


Fig. 2.—Top view of gear mechanism.

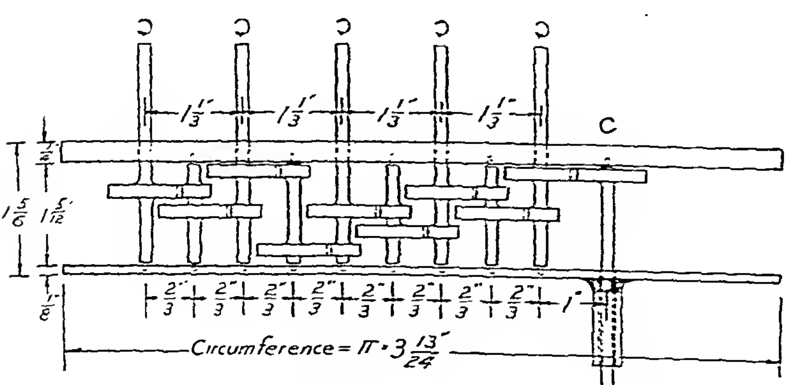


Fig. 3.—Gear mechanism diagrammatically shown in lateral plane.

Base.—The iron base is cast to order as a rectangular box weighing about thirty-five pounds. The bottom is left open and has rubber bumpers screwed up into the rim. The top is covered with a rolled steel plate.

*Decorative Flexboard, Johns-Manville Corp.

Idler Drum and Base.—The idler drum and base are the same as the motor-driven unit, except that the shaft of three-fourth-inch rodding is mounted on a movable plate, which can be slipped back and forth on the base. A heavy, knurled knob connects with this movable plate through a center groove in the top of the base. The drum bases are separated from each other by telescoping pieces of pipe with a threaded attachment for precision adjustments.

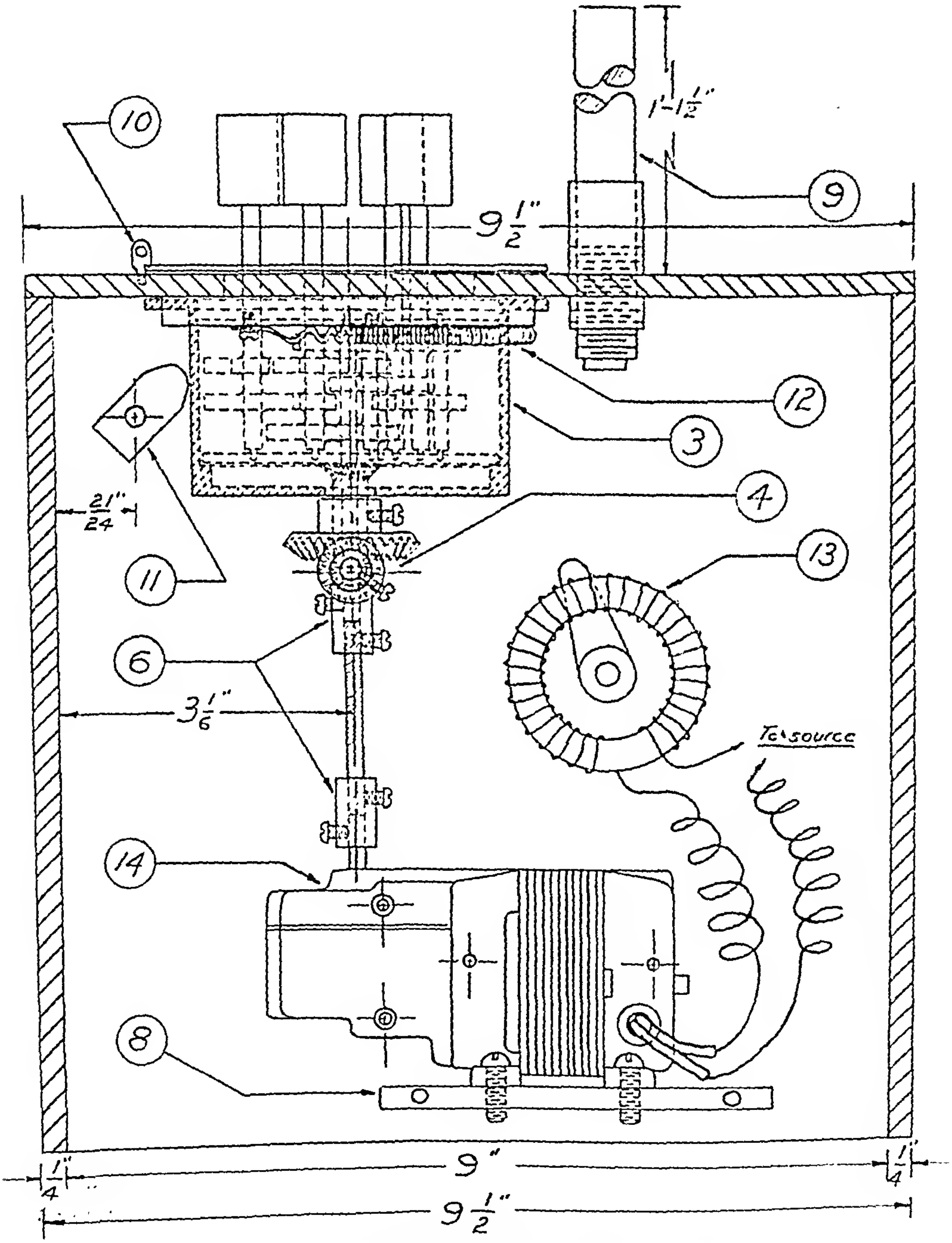


Fig. 4.—Front view (exposed) of gear mechanism.

Gear Box.—The clockwork mechanism is mounted inside a piece of four-inch pipe, and this housing is flanged at the top so as to slide in grooved backets attached to the top plate. A spring maintains tension of the entire housing toward the periphery of the drum. This gear housing is moved back away from the drum periphery by means of a cam shaft operated by a knob in the front of the base. The mechanism is made up of gears with twelve and thirty teeth each. Since each roller drive shaft must be turning in the same direction, two pairs of

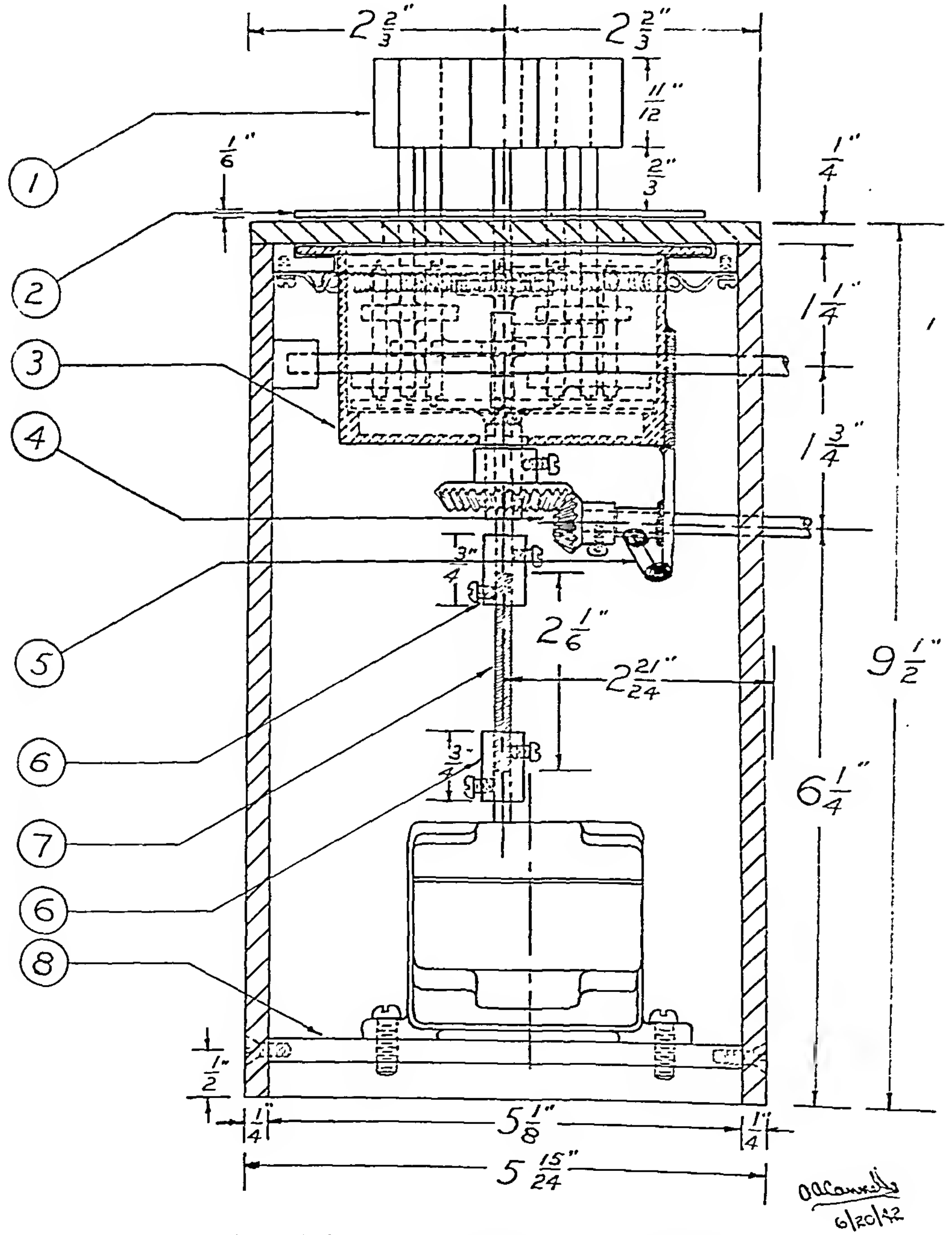


Fig. 5.—Left side view (exposed) of gear mechanism.

such gears are necessary, which, with the gear ratios of 12 and 30, make a speed reduction of 6.25 from one drive shaft to the next. The main drive shaft from the motor enters at the center of the gear housing and drives at a rate of 40 or 80 r.p.m. This low speed subjects the gear mechanism to very little wear and obviates the need for an oil bath or special oiling arrangements. This motor drive shaft is made up partly of a section of flexible shafting since the motor is stationary while the gear box is moved back and forth about one-fourth inch. The gears are mounted on a circular plate which turns smoothly on an offset within the housing and is connected by a threaded and soldered metal tube with a bevel gear placed around the central gear shaft below the gear housing. This bevel gear is turned by a smaller bevel gear which is on a shaft operated by a knob from the front of the base. This bevel gear arrangement is not entirely necessary since there is actually very little interference or inconvenience if the gear platform is simply turned by hand, the rollers and their shafts above the base plate being easily accessible. The correct spacing of the gear centers is necessary in order to minimize "back-lash," which can produce an irregular rate with an unloaded fast drum. The measurements shown in the diagram are approximate and, in setting up the gears, must be calculated more accurately from the known pitch diameter of the gears.

The drive rollers are made of ordinary rubber pressure tubing spiked on to each of the five drive shafts. Considerable variation in size of rollers is possible, which correspondingly varies the drum speeds.

LIST OF MATERIALS AND MANUFACTURERS (EFFECTIVE BEFORE PRESENT EMERGENCY)

Drums. Natural Synthane xx free-machining tubing. 8 inch inside diameter by $\frac{1}{16}$ inch wall. Two 12 inch lengths.
Synthane Corp., Oaks, Pa.

Motor with worm drive reduction.
Speedway Mfg. Co., 1834 South 52nd Ave., Cicero, Ill.

Brass gears. Spur gears. 10 with 12 teeth (No. G163)
10 with 30 teeth (No. G174)
Bevel gears. 1 pair with 18 and 36 teeth (No. G485)
Cat. No. 52. Boston Gear Works, N. Quincy, Mass.

Bases. Two iron castings of 33 lb. at .07
Memphis Machine Works, Memphis, Tenn.

Power rheostat. 75 watt, 300 ohm. No. 27-457
Flexible shafting No. 73-290
On-off switch No. 34-520
Bar knob No. 55076
Fluted knobs No. 55087

Switch plates, dial plates, power cord, crystallizing lacquer, etc.
Cat. No. 107. Allied Radio, 833 W. Jackson Blvd., Chicago, Ill.

Cold rolled steel shafting, mild steel rolled plates, bar iron, spreader pipes, etc.
Lewis Supply Co., Memphis, Tenn.

REFERENCES

1. A. C. Polyphase motors, Product Engineering, March, 1938, p. 123.
2. Ryder: Electronic control circuits for D. C. motors. Electronics, Dec., 1938, p. 20. See also "Variable Speed Through Electronic Control." Product Engineering, June, 1940, p. 244.

INTRAVENOUS ADMINISTRATION OF OXYGEN

F. S. Grodins, M.S., M.D., A. C. Ivy, M.D., Ph.D., and H. F. Adler, Ph.D.
Chicago, Ill.

A RECENT report[1] claims markedly beneficial effects from the intravenous administration of relatively minute amounts of oxygen. It was stated that therapeutic results were obtained with as little as from 0.2 to 0.3 c.c. per kilogram per minute and that this method of administration was more effective than oxygen by any other route. It seemed rather difficult to account for these results, since the amount of oxygen administered is less than 10 per cent (1 to 7 per cent) of the average basal requirement. Moreover, it has previously been reported[2, 3] that intravenous oxygen in larger amounts not only is unable to correct an existing anoxemia but may actually produce an anoxemia when none is present. In view of these facts, the problem has been reinvestigated.

METHODS

Dogs anesthetized with pentobarbital were used in all experiments. Oxygen for intravenous administration was obtained from an ordinary Linde tank fitted with a reducing valve to control the delivery pressure and a needle valve to control the flow. The gas was led through an orifice meter calibrated to measure the rate of flow in cubic centimeters per minute and was administered to the animal through two 27-gauge needles, one in each brachial vein. When oxygen was given by trachea, a closed system with suitable valves to prevent rebreathing was employed. Carotid blood pressure and respiratory movements were recorded on a smoked drum. Blood arterial oxygen was determined by the manometric method of Van Slyke.[4]

RESULTS

Effect on Respiration.—Twelve animals were studied (Table I). In ten animals with intact vagi, intravenous oxygen in amounts as small as 0.21 to 0.64 c.c. per kilogram per minute caused changes in respiration. The rate was most consistently affected, being increased in all cases. The effect on depth was more variable, increases, decreases, or no change occurring with approximately equal frequency. However, the most typical response, at least initially, appeared to be rapid, shallow breathing (Fig. 1). In larger amounts, the respiratory effects were more marked. In three vagotomized dogs, intravenous oxygen produced primarily an increase in depth, the increase in rate being less conspicuous (Fig. 2).

Effect on Blood Pressure.—The blood pressure was affected less consistently than the respiration. In general, small amounts of oxygen produced little

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.
Received for publication, July 29, 1942.

change, whereas larger amounts lowered the pressure. However, there was marked individual variation.

Cardiac Froth.—Small amounts of oxygen (0.20 to 0.35 c.c. per kilogram per minute) did not produce audible cardiac froth. Larger amounts (1.0 to 2.5 c.c. per kilogram per minute) produced audible froth in all cases. This dis-

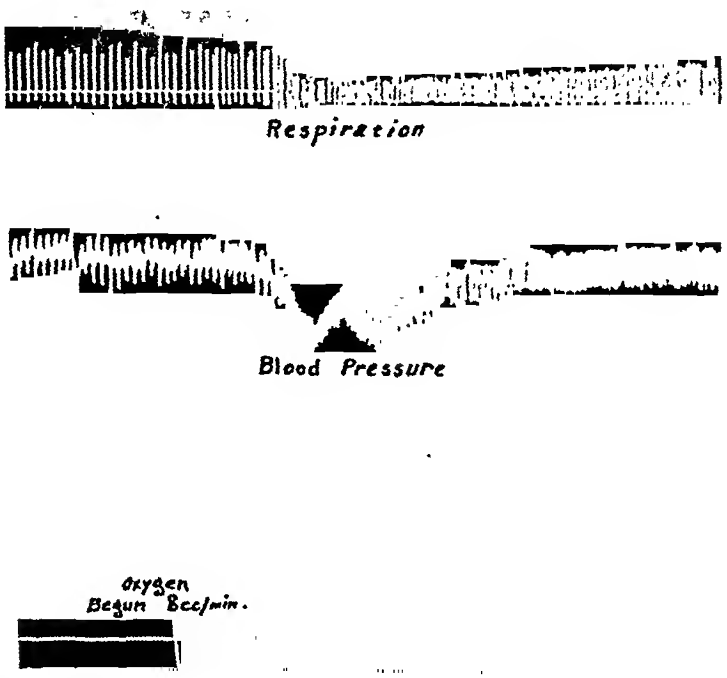


Fig. 1 (Dog 7).—Weight, 12.6 kg. Vagi intact. Intravenous oxygen at 8 c.c. per min. (0.64 c.c. per kilogram per minute). Note typical rapid shallow breathing.

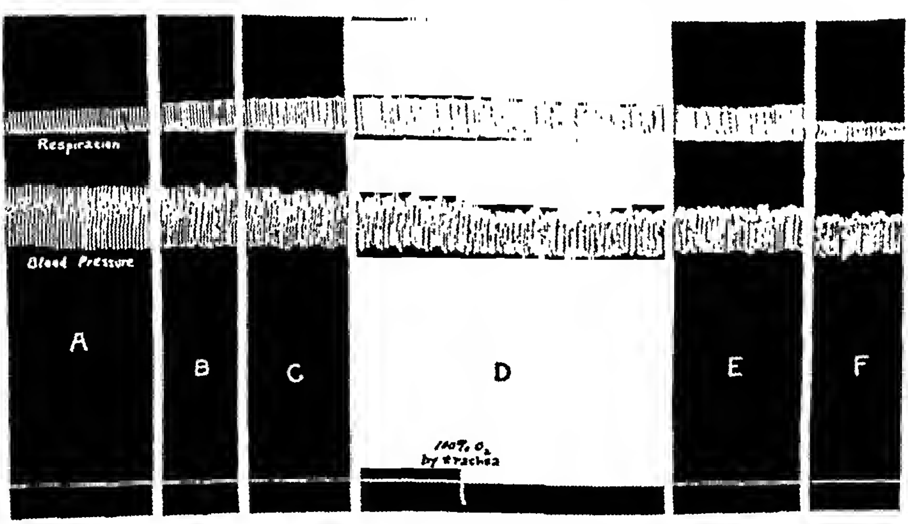


Fig. 2 (Dog 11).—Weight, 13.6 kg. Vagi sectioned. *A*, Control Tracing; *B*, intravenous oxygen 6 c.c. per minute; 0.44 c.c. per kilogram per minute; *C*, intravenous oxygen 10 c.c. per minute; 0.73 c.c. per kilogram per minute; *D*, intravenous oxygen 17 c.c. per minute; 1.2 c.c. per kilogram per minute; 100 per cent oxygen by trachea started at signal; *E*, tracheal oxygen stopped; *F*, intravenous oxygen stopped.

TABLE I

DOG	WEIGHT (KG.)	OXYGEN (C.C. PER MIN.)	OXYGEN (C.C. PER KG. PER MIN.)	RESPIRATION		BLOOD PRESSURE		ARTERIAL O_2 CONTENT		
				RATE	DEPTH	CONTROL	O_2	CONTROL	O_2	PER CENT CHANGE
1	18.2	5	0.27	++	++	128	128			
2	15.9	5	0.31	+++	+++	128	128			
3	13.6	5	0.37	++	++	166	124			
		15	1.10	++	++++	170	114			
4	11.8	2.5	0.21	+++	--	114	86			
		10	0.85	++	--	126	116			
		14	1.18	++	--	120	110			
5	9.0	2	0.22	+++	--	154	118	14.7	9.7	−34
		15	1.67	++++	∓	160	94			
6	13.6	3	0.22	+++	ō	142	142	19.4	20.0	0
		15	1.10	+++	0	140	140	19.4	19.4	0
7	12.6	8	0.64	+++	--	180	142	10.8	8.0	−18.5
		40	3.17	--	--	170	0			
8	10	3.5	0.35	+++	--	132	102	10.8	7.8	−27.8
9	13	6.5	0.50	+++	ō	166	156	21.6	19.7	− 8.8
		30	2.3	+++	ō	156	140	21.6	20.2	− 6.5
100% O_2 by trachea		30	2.3	+++	0	140	160	21.6	23.5	+ 8.8
Vagi cut		6.5	0.50	0	+	164	168			
		30	2.3	+	++	168	154			
10	18	5.5	0.35	++	--	154	134	20.1	17.8	−16.4
11	13.6	6 to 10	0.44 to 0.73	+	++	190	190			
	(vagotomized)	6 to 17	0.44 to 1.2	+	++	190	190			
100% O_2 by trachea		17	1.2	0	0	190	190			
12	18	6 to 16	0.33 to 0.89	+	+	160	142			
	(vagotomized)									
100% O_2 by trachea		16	0.89	-	-	140	120			

appeared quite rapidly (five to ten minutes) after the intravenous oxygen was stopped.

Arterial Oxygen Content.—Blood arterial oxygen was followed in six animals. In five of the six cases, intravenous oxygen in amounts varying from 0.35 to 2.3 c.c. per kilogram per minute decreased the arterial oxygen in amounts varying from 6.5 to 35 per cent.

Fatal Dose.—Dog 7 was killed by 3.2 c.c. per kilogram per minute intravenously.

DISCUSSION

In general, our results are in agreement with those of earlier investigators[2, 3] in so far as changes in respiration, blood pressure, and arterial oxygen are concerned. However, we found these changes to occur with smaller amounts of oxygen than were employed by these workers. From a physiologic viewpoint, several questions arise.

1. *Mechanism of Respiratory Changes.*—The most typical respiratory response to intravenous oxygen was a rapid shallow breathing (Fig. 1). This is the type of response reported by Binger, Brow, and Branch,[5] who produced multiple pulmonary emboli by the intravenous injection of starch granules in dogs, and by Dunn,[6] who performed a similar experiment in the goat. In the latter's experiments, no anoxemia was observed. In Binger's experiments, anoxemia

was produced. However, correction of the anoxemia by the inhalation of 90 per cent oxygen did not change the respiratory response. Freezing of the vagi, however, changed the response to a slow, deep respiration. These authors attributed the rapid shallow breathing to an increased sensitivity of the Hering-Breuer reflex due to the mechanical effects of pulmonary damage.

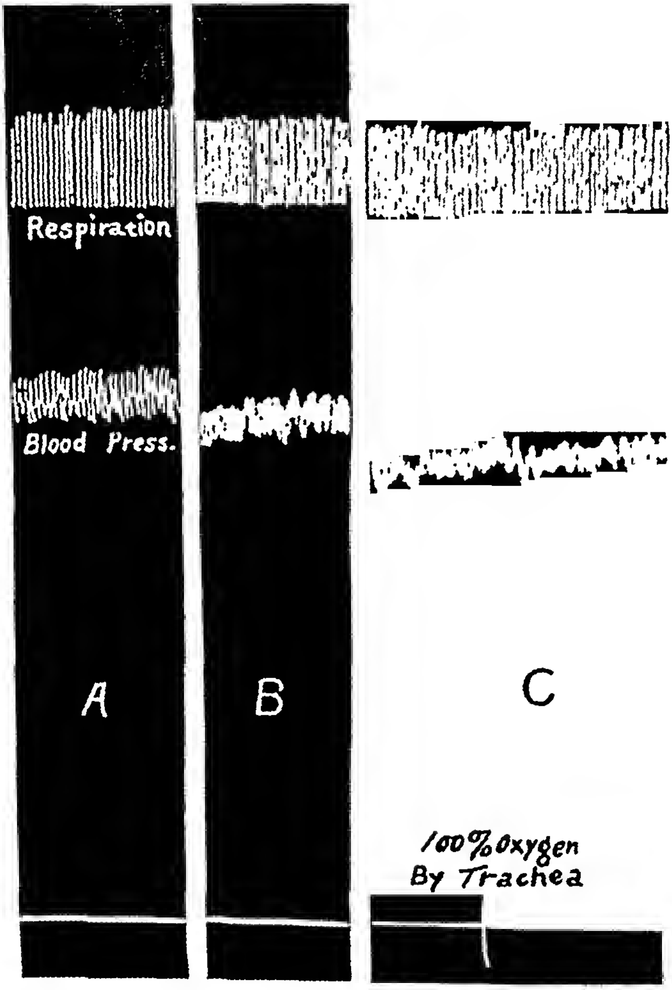


Fig. 3 (Dog 9).—Weight 13 kg. Vagi intact. *A*, Control; *B*, intravenous oxygen 6.5 c.c. per minute; 0.50 c.c. per kilogram per minute; *C*, intravenous oxygen 30 c.c. per minute; 2.3 c.c. per kilogram per minute; 100 per cent oxygen by trachea started at signal.

The respiratory response in our animals with intact vagi also was not primarily due to anoxemia, as demonstrated by Dog 6, in which rapid breathing occurred without the presence of anoxemia, and by Dog 9, in which correction of the anoxemia by the inhalation of 100 per cent oxygen failed to affect the respiratory response (Fig. 3). However, that the response is not entirely a vagal reflex phenomenon arising in the pulmonary tissue is shown by the fact that vagotomy in our animals did not abolish the response entirely, although it changed its character. In these instances, chemical factors were undoubtedly in operation.

From these considerations, the mechanism of the respiratory response to intraveous oxygen apparently depends upon two factors:

1. Pulmonary reflex via the vagi; results in rapid breathing.
2. Carbon dioxide excess and oxygen lack produced by the reduction of the pulmonary capillary bed by multiple gas emboli. According to Schmidt,[7] the typical response to these chemical stimuli is an increased depth of respiration.

In intact animals, the first factor is most important; in vagotomized animals, the second factor is dominant. For example, in Dog 11, which was vagotomized, 100 per cent oxygen by trachea only slightly decreased the respiratory response (Fig. 2). In Dog 12, however, (also vagotomized) 100 per cent tracheal oxygen markedly diminished the hyperpnea (Fig. 4). In the former case, it can be assumed that the respiratory drive was primarily centrogenic, produced by excess carbon dioxide on the respiratory center. In the second animal, which was very deeply anesthetized, the respiratory drive was chiefly reflexogenic due to the effect of anoxemia on the carotid sinus. This latter case is exactly analogous to the "oxygen inhibition" reported by Marshall and Rosenfeld[8] to occur in deeply nembutalized dogs.

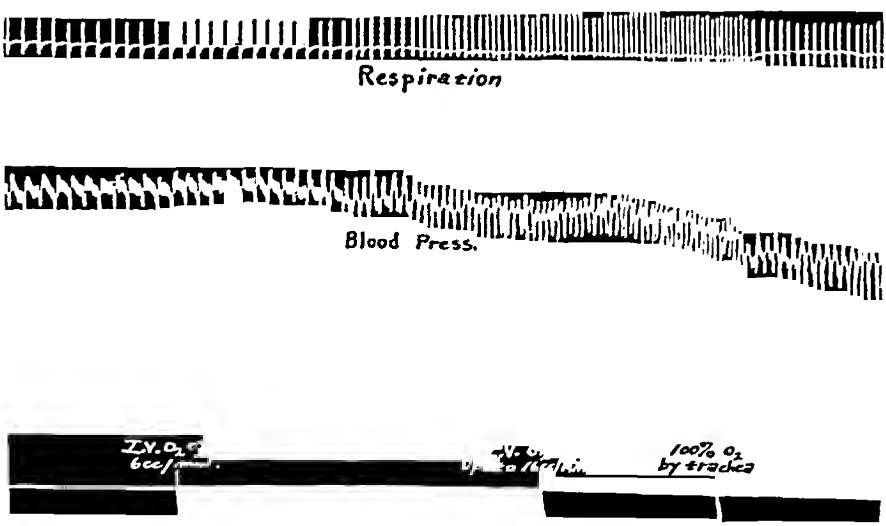


Fig. 4 (Dog 12).—Weight 18 kg. Vagi sectioned. Note inhibiting effect of 100 per cent oxygen by trachea on hyperpnea caused by intravenous oxygen.

2. *Mechanism of the Anoxemia.*—This is probably due to an anoxic anoxia caused by a reduction in the pulmonary capillary bed by multiple gas emboli. The oxygen supplied by vein cannot compensate for the reduced pulmonary supply. With larger amounts, decreased efficiency of the pulmonary circulation due to air bubbles in the right heart is undoubtedly important.

From a therapeutic viewpoint, our results fail to support the claims of Viegler.[1] Intravenous oxygen was not only unable to correct an existing anoxia produced by nembutal anesthesia, but actually increased the anoxemia. That nembutal anoxemia can be corrected by oxygen inhalation has been reported by Schnedorf[9] and McClure and associates,[10] and confirmed by us in Dog 9.

COMMENT

It would appear that if intravenous oxygen therapy will ever prove to be efficient, some way will have to be found so that significant amounts can be administered without causing bubble formation.

SUMMARY

1. Intravenous oxygen in relatively small amounts causes marked respiratory changes, may cause a fall in blood pressure, and usually reduces the arterial oxygen content.

2. The mechanism of the respiratory response and the anoxemia has been discussed.

3. Our results indicate that the intravenous administration of oxygen at rates varying from 0.35 to 2.3 c.c. per kilogram per minute is not only unable to correct an existing anoxemia, but actually aggravates the condition.

REFERENCES

1. Ziegler, E. E.: Intravenous Administration of Oxygen, J. Lab. & Clin. Med. 27: 223, 1941.
2. Bourne, G., and Smith, R. G.: The Value of Intravenous and Intraperitoneal Administration of Oxygen, Am. J. Physiol. 82: 328, 1927.
3. Dick, M.: Respiratory and Circulatory Responses to Intravenous Oxygen and Their Relation to Anoxemia, Am. J. Physiol. 127: 228, 1939.
4. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, vol. 2, Baltimore, 1932, Williams & Wilkins Co.
5. Binger, C. A. L., Brow, G. R., and Branch, A.: Experimental Studies on Rapid Breathing. I. Tachypnea, Independent of Anoxemia, Resulting From Multiple Emboli in the Pulmonary Arterioles and Capillaries, J. Clin. Investigation 1: 127, 1924; Experimental Studies on Rapid Breathing. II. Tachypnea, Dependent Upon Anoxemia, Resulting From Multiple Emboli in the Larger Branches of the Pulmonary Artery, J. Clin. Investigation 1: 155, 1924.
6. Dunn, J. S.: The Effects of Multiple Embolism of the Pulmonary Arterioles, Quart. J. Med. 13: 129, 1919.
7. Schmidt, C. F.: Macleod's Physiology in Modern Medicine, St. Louis, 1941, The C. V. Mosby Co., chap. 28.
8. Marshal, E. K., Jr., and Rosenfeld, M.: Depression of Respiration by Oxygen, J. Pharmacol. & Exper. Therap. 57: 437, 1936.
9. Schnedorf, J. G.: Oxygen Therapy in Reactions Following Barbiturate Anesthesia and Cisternal Intervention, Surg., Gynec. and Obst. 69: 305, 1939.
10. McClure, R. D., Hartman, F. W., Schnedorf, J. G., and Schelling, V.: Anoxia. A Source of Possible Complications in Surgical Anesthesia, Ann. Surg. 110: 835, 1939.

AN EFFICIENT APPARATUS FOR THE PREPARATION OF PARENTERAL SOLUTIONS

ALEXANDER G. KELLER, B.Sc., PHILADELPHIA, PA.

AS THE importance of parenteral solutions has grown to such a magnitude during the last ten years, many hospitals have transferred the preparation of these solutions from the operating room staff to the laboratory department, where a special room and personnel are used for the work.

This transfer occurred in our hospital approximately eight years ago, and from one small room we recently expanded to two rooms with a total area of 500 square feet. The rooms are adjoining, one for the cleaning of the glassware and the other for the preparation and bottling of the solutions.

PART I

The cleaning of the glassware is conducted in several steps: (1) the glassware is boiled for fifteen minutes in a detergent solution in a tank; (2) it is thoroughly rinsed with hot tap water for ten minutes; and (3) it is rinsed with distilled water for three minutes (Fig. 1).

For a tank in which to heat the glassware, we use an enameled bathtub. The tub is filled to the overflow level, capacity fifty gallons, with tap water. A wetting agent* is added to remove the hardness of the water and to prevent the formation of a precipitate when the trisodium phosphate is added. Sufficient phosphate is added to give a concentration of approximately 0.5 per cent.

After boiling the glassware the liquid is drained from the tank and the glassware is transferred to the rinsing apparatus. This consists of a shallow bathtub with two pipes, lying lengthwise, on the bottom of the tub. Each pipe has eight vertical nozzles. A wooden rack rests on the pipes, holding a flask mouth down, over each nozzle. By opening a valve, hot tap water enters the two pipes and nozzles and sprays the inside of each flask. The outside of each flask is washed by a rubber tube operated manually by the technician. Finally by closing the hot tap water valve and opening an adjacent valve, distilled water is admitted through the system, and any tap water adhering to the glassware is removed.

In order to have the distilled water under sufficient pressure for the rinsing, we use a vertical style autoclave to which is attached an electric pressure pump.

When the rinsing is completed, each flask is removed and a piece of parchment paper, freshly rinsed with distilled water, is placed over the mouth.

From the Biochemical Laboratories of the Graduate Hospital, University of Pennsylvania.
Received for publication, Sept. 23, 1942.

*Prepared by the Amalgamated Chemical Co. under the trade name Litho Grains No. 2.

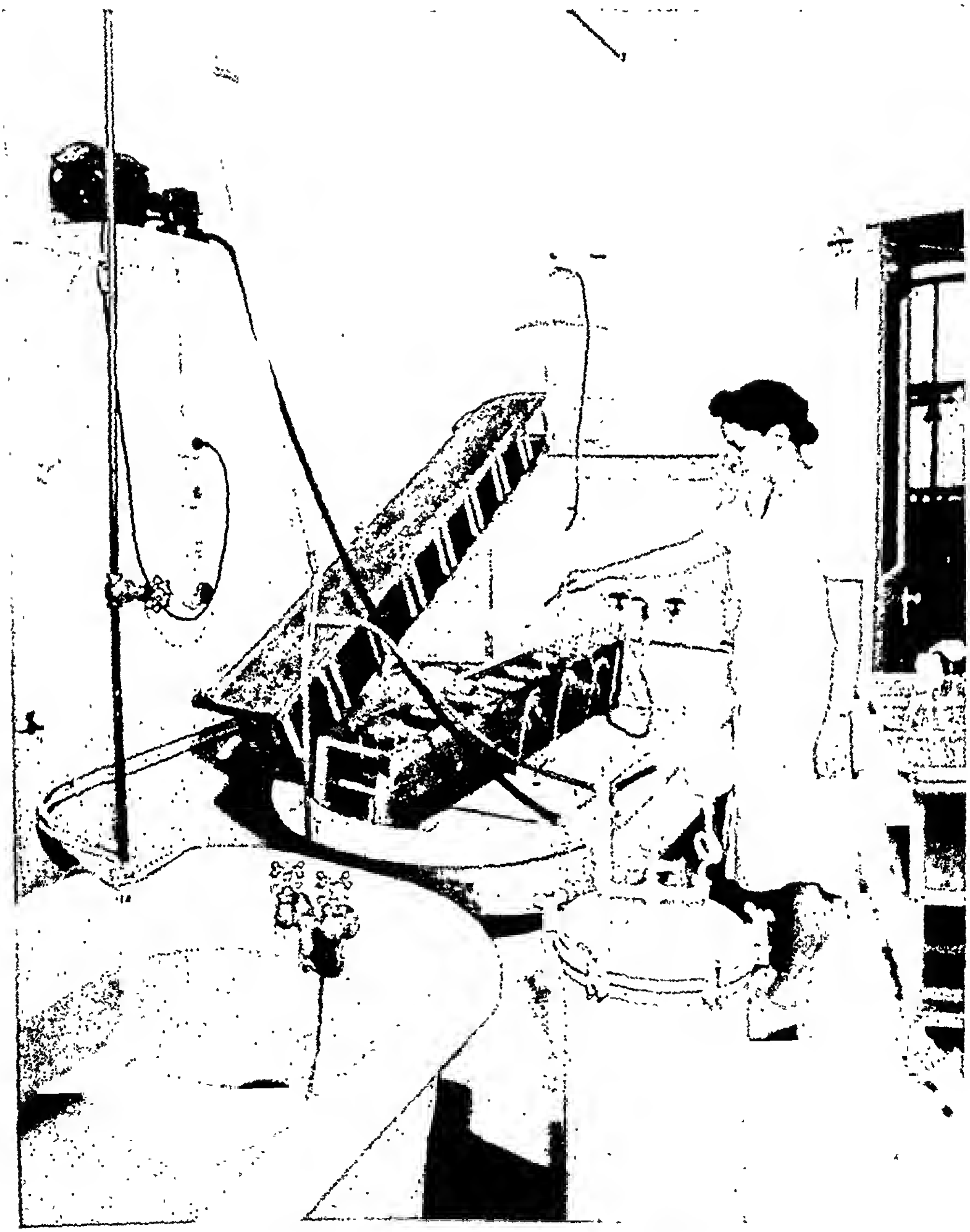


Fig. 1.

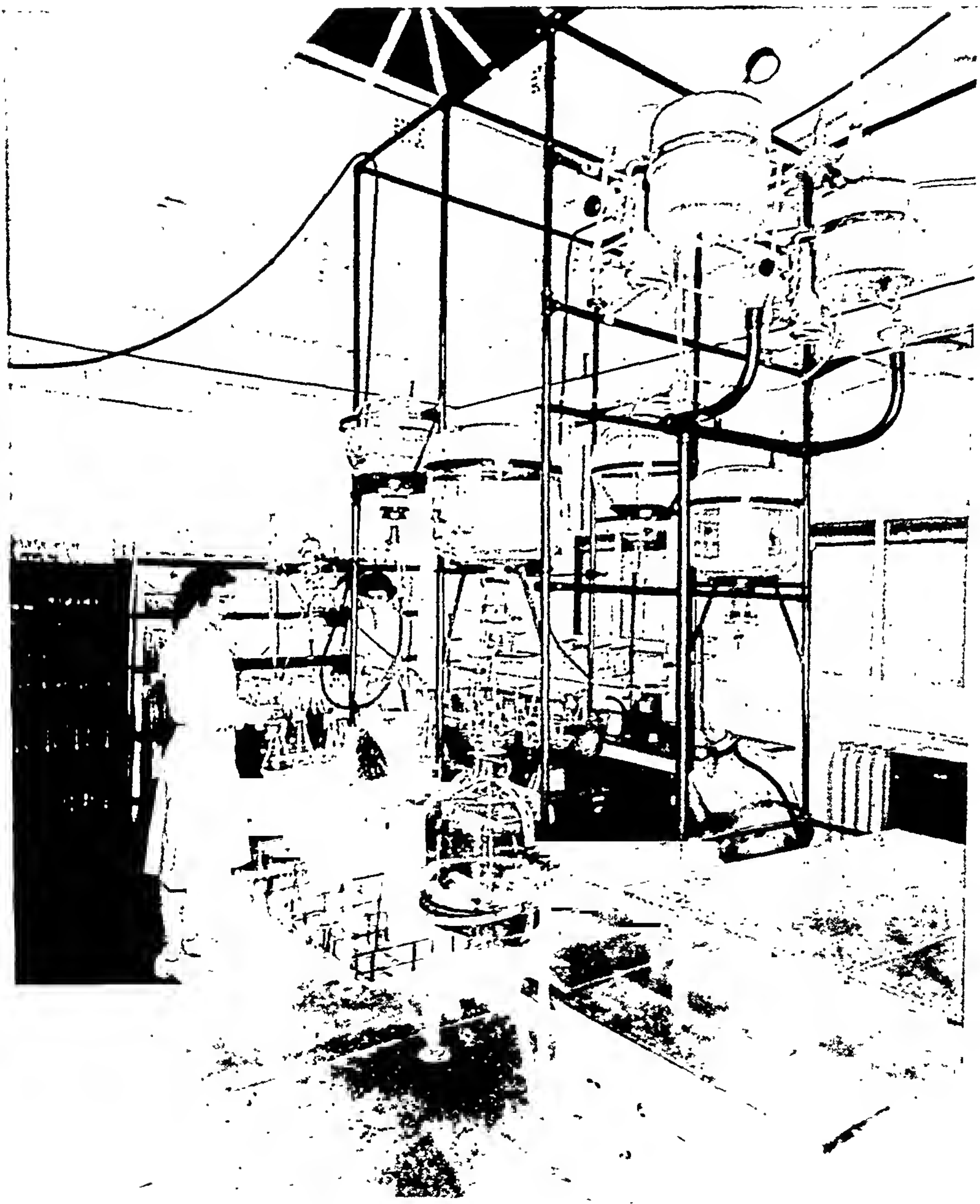


Fig. 2.

PART II

Water is distilled fresh each day by two* five-gallon-per-hour steam-heated stills,* mounted on an iron framework approximately ten feet from the floor (Fig. 2).

The distillate is collected in an inverted twelve-gallon pyrex carboy, mounted in the framework at a level below that of the stills. Directly below this inverted carboy is a second twelve-gallon carboy held in a metal harness attached by a swivel arrangement to the upright on each side. This permits the carboy to be inverted when desired.

The constituents for a parenteral solution are delivered into this lower carboy. The fresh distilled water enters from the upper carboy through a glass tube. In the center of this lower carboy, held in place by the rubber stopper, is a graduated glass tube to indicate the volume of liquid.

Mixing is accomplished by drawing air through the liquid, using a water suction pump. The air is cleaned before entering the liquid by passing through soda lime, weak sulfuric acid, and distilled water.

After mixing, the liquid is drawn by suction through glass tubing to a 50 L. spherical flask adjacent to the upper carboy. In this transit the liquid is filtered through a fritted glass pyrex filter, medium porosity. As a partial vacuum must be created, a spherical flask is used in preference to a standard-shaped bottle. The perforated rubber stopper has a metal disk attached to it on the outside of the flask to prevent the stopper from being sucked in should the vacuum become too great.

The partial vacuum is created by a water suction pump. A glass check valve is placed in the line betwen the pump and the flask to prevent backfire. A gauge is also in the line so the number of inches of vacuum can readily be seen. The vacuum should not exceed fifteen inches. As soon as the spherical flask is half full, the water suction pump is stopped and the vacuum permitted to decline voluntarily. When room pressure is reached, the liquid is permitted to enter the dispensing apparatus.

The dispensing apparatus is essentially a large burette with an overflow. It is operated with pinchcocks and rubber tubing in place of a glass stopcock.

The previously cleaned Erlenmeyer flasks are filled to half their capacity from the dispensing apparatus. The parchment paper cap is replaced, and an additional pleated paper cap or hood, bearing the date of manufacture, is placed over the parchment paper.

After a close inspection to ascertain the presence of any foreign particles in the liquid, two wire ties are placed around the neck of the pleated paper cap and twisted tight.

All solutions are autoclaved the same day they are made. Bactriologic controls are placed in the autoclave at the same time.

A duplicate unit of collection bottle, mixing bottle, spherical flask, etc. is parallel to the first unit described. This permits two different solutions, or two batches of the same solution, to be made simultaneously. The distillate can be delivered to either receiving bottle by opening or closing a pinchcock.

*Made by American Sterilizer Co.

This equipment will produce large quantities of parenteral solutions per day. The iron framework was built by the hospital maintenance department at a comparatively small cost.

No paper filters are used.

The solution is protected from exposure to the air to a great degree.

CONCLUSION

An efficient as well as economical apparatus is described for preparing parenteral solutions.

AN ELECTRIC LANTERN SLIDE POINTER

NELSON A. MURRAY,* M.D., ROCHESTER, MINN.

THIS useful piece of apparatus can be made from odds and ends found in almost any laboratory and will well repay one for the few minutes spent in its construction. The pointer which I made was constructed from the battery case of a Mendelsohn flashlight gun, an adapter for the flashlight bulb, a cardboard mailing carton, two round wooden micro cover slip boxes, and a 7 diopter spectacle lens (Fig. 1). A 7 diopter lens is of approximately 14.28 cm. focal length. This focal length was chosen for no other reason than that it is a convenient length. A lens of 6 or 8 diopters will work equally well.

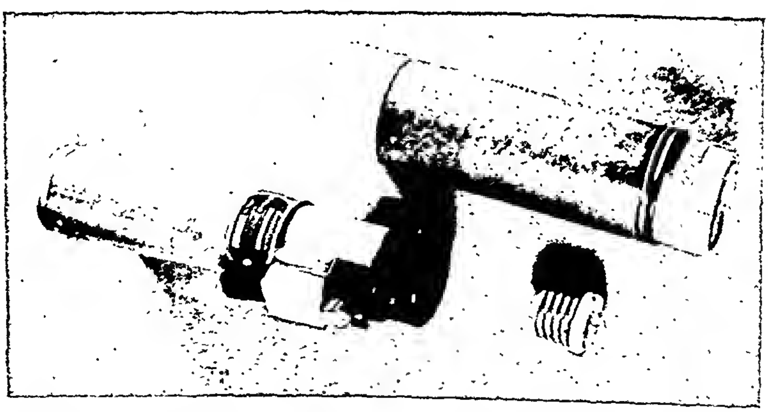


Fig. 1.—Material used in constructing the electric lantern slide pointer.

The lens is placed in front of the flashlight bulb so that the distance between the lens and the light source is slightly greater than the focal length of the lens (Fig. 2). The actual distance can be calculated readily from the standard lens formula. In order to set the lens for a spot at fifteen feet (500 cm.), calculations are as follows: $1/p + 1/q = 1/F$. When $p = 500$ cm. and $F = 14.28$

Received for publication July 27, 1942.

*Fellow in Pathology, Mayo Foundation.

cm., then q = 14.70 cm. Setting the lens at 14.70 cm. from the light source, a small, sharp spot will be focused at approximately fifteen feet. The pointer is adjustable so that by sliding the lens mount (coverslip box) in and out, the distance between the pointer and a sharp focused spot may be varied at will.

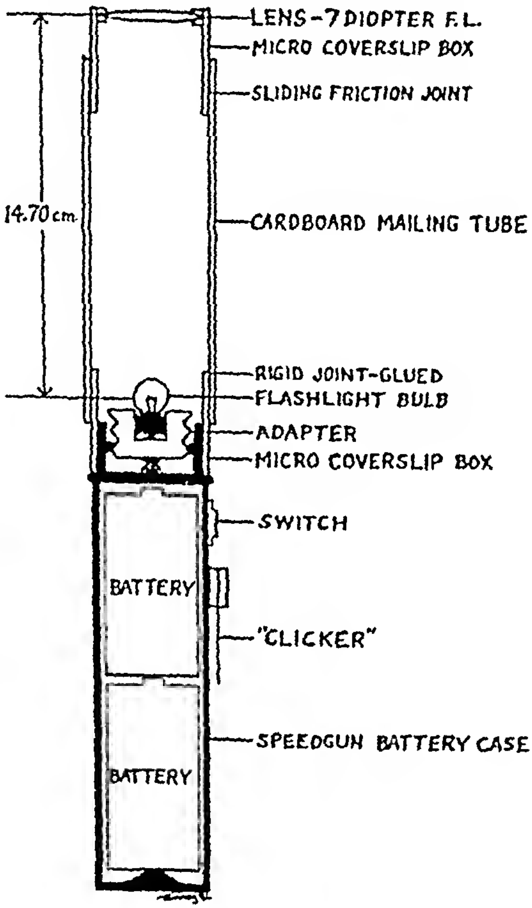


Fig. 2.—Diagram of the electric lantern slide pointer.

A piece of spring steel (slightly bent in the middle by tapping with a machinist's hammer) 1½ by ¾ by 1/32 inch (3.8 by 1.9 by 0.08 cm.) attached to the battery case, as illustrated, emits a "clicking" sound when pressed with the thumb and affords a convenient signal to the lantern operator to change to the next slide. Colored cellophane may be placed behind the lens if a colored spot is desired.

SS AGAR FOR THE ISOLATION OF EBERTHELLA, SALMONELLA, AND SHIGELLA GROUPS FROM FECES*

Compared With MacConkey and Bismuth Sulfite Agars and Tetrathionate Broth Followed by MacConkey Agar†

Mollie Mollov, M.S., Jeanette E. Winter, B.A., and Philip Steinberg, B.S., Jamaica, N. Y.

THE problem of routine isolation of Eberthella, Salmonella, and Shigella organisms from fecal specimens is one of vital importance from a public health standpoint. Detection of the etiologic agents is essential in the diagnosis of enteric bacillary disease and in the recognition of carriers. An efficient selective medium or combination of media is most important for the successful isolation of the incriminating organisms. Many types of media have been devised to facilitate the isolation of these organisms. Few, if any, have proved to be entirely satisfactory for all the enteric pathogens. The medium was often insufficiently inhibitive to prevent overgrowth of the coliform bacilli, or it failed to support the growth of some of the more fastidious organisms.

Liefson,[1] in 1935, devised the desoxycholate-citrate agar as a selective, inhibiting medium which enhanced the isolation of the Flexner type of *Shigella paradysenteriae* bacilli but which inhibited the Shiga, Sonne, dispar and alkalescens strains to a great extent. Paulson,[2] in 1937, found desoxycholate-citrate agar superior to the Endo and eosin-methylene blue agars in bowel disorders. Hardy and Watt[3, 4] confirmed the superiority of the desoxycholate-citrate medium in their experience with their Flexner dysentery cases. They mention, further, that the differential MacConkey agar compares favorably with the Endo and eosin-methylene blue but not with the desoxycholate-citrate agar in the isolation of Flexner dysentery bacilli. Cooper and associates[5] also conclude that MacConkey is superior to Endo and eosin-methylene blue but that desoxycholate-citrate is superior to all three for Flexner and also for Sonne dysentery isolations. Sellers, Morris, and Reynolds,[6] in 1934, found bismuth sulfite agar better than Endo for isolating *Eberthella typhosa*. Gunther and Tuft[7] concluded that bismuth sulfite is superior to Endo, eosin-methylene blue, and desoxycholate-citrate agar in the isolation of *E. typhosa* from urine and feces. Jones[8] found brilliant green-eosin agar superior to MacConkey for dysentery bacilli. Desoxycholate-citrate agar was found to be much better than MacConkey for isolating dysentery bacilli by Irons and co-workers[9] in 1939. Difco Laboratories, Inc.,[10] in 1939, revised their formula for MacConkey agar to support the growth of all the dysentery as well as the typhoid and Salmonella organisms.

*From the Bacteriology Department and the Department of Pathology, Queens General Hospital, Jamaica, Long Island, N. Y.

Received for publication, July 29, 1942.

†SS, bismuth sulfite, and MacConkey agars and tetrathionate broth used in this study were products purchased from the Difco Laboratories.

Studies with SS (Shigella-Salmonella) agar were first reported by Mayfield and Gober[11] 1940. They found SS agar to be as efficient as desoxycholate-citrate for the isolation of dysentery bacilli. They mention, further, that the pathogenic and nonpathogenic* colonies were more clearly defined on the SS medium. Rose and Kolodny[12] confirmed these findings in their studies of Flexner and Sonne dysentery cases. Cooper, Keller and Glesne,[13] in 1942, reported the superiority of SS and desoxycholate-citrate agars over the MacConkey agar in cases caused by *Shigella paradysenteriae* Flexner. They stated, further, that MacConkey was slightly more efficient in isolating Sonne dysentery organisms and also, that although the SS and desoxycholate-citrate agars were equally efficient in the isolation of Flexner dysentery bacilli, a simpler and more distinct differentiation on SS of proteus organisms were present, thus eliminating need for many subcultures.

Several types of *liquid media* have been described to enhance the growth and facilitate the isolation of pathogenic intestinal organisms. In 1936 Leifson[14] described the combination of selenite F enrichment and desoxycholate agar for the isolation of typhoid bacilli. The sodium acid selenite salt favors the growth of typhoid and paratyphoid bacilli and inhibits the coliform organisms. In 1923 Mueller[15] demonstrated that tetrathionate broth, also a selective fluid medium, inhibited the coliform bacilli and permitted the growth of typhoid and paratyphoid bacilli. Kaufman[16] in 1936 increased his positive findings of *E. typhosa* and members of the Salmonella group with this medium. In 1939 Difco Laboratories, Inc.,[17] recommended the use of their tetrathionate broth for enriching stool specimens for the isolation of typhoid, paratyphoid, and dysentery bacilli.

We wish to offer our observations on 501 fecal specimens cultured on SS and MacConkey agars and tetrathionate broth and to compare the findings of our positive cases with the media employed. We wish to report the efficiency of SS agar in isolating many members of the Shigella and Salmonella groups and many strains of *E. typhosa*.

We would also like to mention our results with SS, bismuth sulfite, and MacConkey agars in the isolation of *E. typhosa* from feces in a few known cases of typhoid and one known typhoid carrier.

MATERIAL USED

Fecal cultures of dietary workers, nurses, and interns were examined routinely to rule out carriers of intestinal pathogens. Fecal specimens of hospital cases showing bowel disorders, as diarrhea and the like, were also cultured. In no instance in this study had we any indication in the primary culture of the patient as to whether the specimen contained enteric pathogens. Where no pathogens were isolated from any of the media employed, the case was considered negative either because of the inadequacy of our media or the absence of organisms. The specimen that showed enteric pathogens on all or any of the media employed was considered positive. We wish to compare the results of these positive cases for the evaluation of the media used.

*In this paper, by pathogenic organisms we refer to those belonging to the Eberthella, Salmonella, and Shigella groups.

TECHNIQUE

Fresh fecal specimens were sent to the laboratory in clean paper cups fitted with tight covers. Inoculations were made onto one plate each of SS agar and MacConkey agar and into one tube of tetrathionate broth. Each medium was inoculated separately. A large inoculum was placed on the SS plate and streaked freely over the surface. A large inoculum was put into the 10 c.c. of tetrathionate broth to which was added, just before use, 0.2 c.c. of an iodine solution, which was made up of 5 Gm. of potassium iodide and 6 Gm. of iodine to 20 c.c. of distilled water.* A much smaller inoculum was streaked on the MacConkey agar, often diminishing the inoculum, after making two to three streaks over part of the surface by cutting through the agar with the platinum wire at the outermost edge of the medium and then continuing to streak over the unused surface of the plate. All three media were incubated at 37° C. The agar plates were read after eighteen to twenty-four hours, and the number and character of the nonlactose-fermenting colonies on each solid medium were noted and recorded. Two to four of each type of nonlactose fermenting colony were fished to Krumweide sugar slants from each plate. The tetrathionate broth was streaked to MacConkey agar after incubation. This plate was in turn incubated overnight and treated as the initial agar plates. One Krumweide triple sugar slant from each case, giving typhoid, paratyphoid, or dysentery reactions was studied in detail. The other Krumweide slants were kept at icebox temperature until the identification of the slant was completed. Subcultures were made to phenol red broth fermentation tubes (lactose, saccharose, dextrose, mannitol, maltose, sorbitol, dulcitol, salicin). Tryptone broth was inoculated for indol production. Motility tests were done on young cultures from nutrient broth. Motility test medium, a semisolid agar† was used when broth cultures failed to show motility. Fermentation tests were read at twenty-four and seventy-two hours and then intermittently for ten days. All Shigella organisms were idenified serologically by us and checked by the New York City Health Department. The Salmonella organisms were identified serologically by the International Salmonella Committee at the Beth Israel Hospital, New York. All final identifications were based on cultural and serologic findings. The organism from one Krumweide slant from each specimen was studied in detail. However, if the first Krumweide triple sugar slant chosen gave atypical reactions, another slant, fished at the same time from another suspicious colony, was carried through the same procedure detailed above.

In our studies of the known typhoid cases and the known typhoid carrier, in addition to the SS and MacConkey media, a bismuth sulfite agar plate was used. A large inoculum was streaked freely over the entire surface of the medium, and the plate was incubated at 37° C. for forty-eight hours. This plate was then examined, two to four characteristic black colonies were fished to Krumweide triple sugar slants, and the same procedure as described above was followed.

RESULTS

Five hundred and one unknown fecal specimens were examined. Three hundred and thirty-three were from dietary workers, nurses, and interns ap-

*Recommended by the Difco Laboratories, Inc.
†Difco Laboratories, Inc.

parently in good health. One hundred and sixty-eight were from hospital patients with possible intestinal disorders. Of the 333 specimens mentioned above, fourteen specimens were positive. These were isolated from five individuals who were now labelled carriers of typhoid, Sonne, atypical dysentery one case each), and alkalaseens (two cases each). For purposes of studying the efficiency of the culture media employed, we are listing each specimen as a separate case. Of the 168 hospital cases, twenty-six specimens were positive from sixteen cases. Table I lists the types of organisms in the Eberthella, Salmonella, and Shigella groups found to grow on SS agar and also compares the nonlactose fermenting colonies noted in each case, quantitatively, on each type of medium used in this study. It is significant to note that *of the forty positive cases listed, thirty-four were isolated on SS agar only, twenty-seven in tetrathionate broth followed by MacConkey agar, and seventeen on the MacConkey*

TABLE I

COMPARATIVE RESULTS WITH SS AND MACCONKEY AGARS QUALITATIVE RESULTS WITH TETRATHIONATE BROTH

CASE OR CARRIER	ORGANISMS	NUMBER OF PATHOGENIC COLONIES ON		TETRA
		SS	MAC	
1. Case (S. Z.)	E. typhosa	Many	None	Positive
2. Case (R. W.)	E. typhosa	Many	None	No growth
3. Carrier (G. C.)	E. typhosa	Overgrown	Overgrown	Positive
4. Carrier (G. C.)	E. typhosa	Many	Many	Positive
5. Carrier (G. C.)	E. typhosa	Numerous	Overgrown	Positive
6. Carrier (G. C.)	E. typhosa	Numerous	Overgrown	Positive
7. Carrier (G. C.)	E. typhosa	Many	Overgrown	Positive
8. Carrier (G. C.)	E. typhosa	Many	Few	Positive
9. Case (H. D.)	E. typhosa	Few	Numerous	No growth
10. Case (H. D.)	E. typhosa	Many	Overgrown	Negative
11. Case (H. D.)	E. typhosa	Many	Overgrown	Negative
12. Case (H. D.)	E. typhosa	Many	None	Positive
13. Case (L. G.)	E. typhosa	Few	Many	Positive
14. Case (L. G.)	E. typhosa	Numerous	Numerous	Positive
15. Case (L. G.)	E. typhosa	Many	Numerous	Positive
16. Case (L. G.)	E. typhosa	None	Few	Negative
17. Carrier (G. A.)	Sh. Sonne	Many	None	Negative
18. Carrier (D. M.)	Sh. Sonne	Few	Few	Positive
19. Carrier (D. M.)	Sh. Sonne	Occasional	None	Negative
20. Case (J. G.)	Sh. Flexner	Many	None	No growth
21. Case (J. G.)	Sh. Flexner	Few	None	No growth
22. Case (J. G.)	Sh. Flexner	Few	None	Negative
23. Case (W. M.)	Sh. Flexner	Many	None	Negative
24. Carrier (B. M.)	Sh. alkalescens	Many	Few	Negative
25. Carrier (B. M.)	Sh. alkalescens	Few	Few	Positive
26. Carrier (X. M.)	Sh. alkalescens	Many	Occasional	Positive
27. Carrier (X. M.)	Sh. alkalescens	Few	None	Positive
28. Carrier (G. R.)	Sh. atypical	None	None	Positive
29. Case (G. A.)	Sh. atypical	Many	None	Negative
30. Case (F. H.)	S. schottmülleri	Numerous	Few	Positive
31. Case (E. D.)	S. montevideo	None	None	Positive
32. Case (T. J.)	S. newport	Many	Few	Positive
33. Case (M. S.)	S. cholerae suis var. Kuzendorf	Many	Few	Positive
34. Case (W. T.)	S. typhi murium	Many	Few	Positive
35. Case (W. Z.)	S. oranienberg	Overgrown	Overgrown	Positive
36. Case (W. Z.)	S. oranienberg	Many	Overgrown	Positive
37. Case (W. Z.)	S. oranienberg	Occasional	Overgrown	Positive
38. Case (B. E.)	S. oranienberg	Many	Many	Positive
39. Case (G. C.)	S. urbana	Few	Occasional	Positive
40. Case (C. W.)	S. derby	None	None	Positive

agar. In eight cases, the SS agar only was positive, and in five instances, only the tetrathionate picked up the intestinal pathogens. In two of these five cases, the SS and MacConkey agars were overgrown by coli because too large an inoculum was used. In one case only was the MacConkey plate alone positive. It will be noted further that SS was as efficient or superior to MacConkey for all but one case of typhoid and for many types of Shigella and Salmonella. Table II illustrates the total number and percentage of positive cultures on the various media used, and Table III illustrates the number and percentage of positive findings with the various combinations of the three media.

TABLE II

NUMBER OF PERCENTAGE OF POSITIVE CULTURES WITH THE VARIOUS MEDIA

ORGANISMS	TOTAL NUMBER	NUMBER OF POSITIVE CULTURES ON		
		SS	MAC	TETRA
E. typhosa	16	14	7	11
Sh. Sonne	3	3	1	1
Sh. Flexner	4	4	0	0
Sh. alkalescens	4	4	3	3
Sh. atypical	2	1	0	1
S. schottmülleri	1	1	1	1
S. montevideo	1	0	0	1
S. newport	1	1	1	1
S. cholerae suis var Kuzendorf	1	1	1	1
S. typhi murium	1	1	1	1
S. oranienberg	4	3	1	4
S. urbana	1	1	1	1
S. derby	1	0	0	1
Total positive cultures	40	34	17	27
Per cent of positive cultures	100	85	44.4	67.4

TABLE III

COMPARISON OF TOTAL POSITIVE FINDINGS WITH EACH MEDIUM AND WITH VARIOUS COMBINATIONS OF MEDIA

	TOTAL POSITIVE	PER CENT POSITIVE
SS agar	34	85.0
SS plus MacConkey agar	35	87.5
SS plus tetrathionate broth	39	97.5
MacConkey agar	17	44.4
MacConkey agar plus tetrathionate broth	29	72.5
Tetrathionate broth	27	67.4
SS and MacConkey agar and Tetrathionate broth	40	100.0

It is of interest that SS agar aided in the diagnosis of 85 per cent of the positive cases, and the combined use of SS agar and tetrathionate broth allowed successful isolations in 97.5 per cent of the studied cases. In contrast, we find MacConkey agar successful in the isolation of 44.4 per cent of the cases, and the combined use of MacConkey agar and tetrathionate broth successful in 72.5 per cent of the cases studied. It is of significance that SS agar in conjunction with tetrathionate broth allowed us to diagnose all but one of these forty cases.

DISCUSSION

We have run the gamut of culture media for the detection of intestinal pathogens from feces in our laboratory. As a differential medium, MacConkey agar was found to be superior to eosin-methylene blue and Endo (the two most

commonly used types of media). This is in agreement with the published results of Hardy and Watt, Cooper and co-workers, and others. It is the most clear-cut differential medium so far described which supports the growth of all intestinal pathogens and coliform bacilli. The coli colonies are brick red surrounded by a zone of precipitated bile. The colonies of Eberthella, Salmonella, and Shigella are uncolored and transparent and appear as clearing areas in the precipitated bile, caused by the coliform organisms. This makes possible a clear-cut reading when the plate is not overcrowded.

As a selective inhibitive medium we have found the SS agar superior to desoxycholate-citrate (unpublished). This is also in agreement with Mayfield and Gober and others. The SS medium gives maximum inhibition of coli with minimum restriction of the intestinal pathogens. It was found to support the growth of strains of Flexner, Sonne, alkalescens, and an atypical dysentery organism, many strains of *E. typhosa,* and a long list of many types of Salmonella, such as *S. newport, S. montevideo, S. cholerae suis* var. Kunzendorf, S. typhi murium, S. oranienberg, *S. urbana, S. schottmülleri,* and *S. derby.* This is an obvious advantage. Desoxycholate-citrate agar is recommended for the isolation of Flexner and Schmidt's dysentery bacilli but which restricts the growth of Shiga, Sonne, and alkalescens types of paradysentery bacilli. Bismuth sulfite is recommended to be superior to desoxycholate-citrate, eosin-methylene blue, and Endo agars for the isolation of *E. typhosa.*

In a very small series of known cases and one carrier of typhoid we have compared bismuth sulfite, SS and MacConkey agars. In all but one case have we found SS as efficient or superior to bismuth sulfite agar in isolating *E. typhosa* from feces, and in that case the MacConkey agar was also positive. Further study with these three media will be reported in a subsequent paper.

In SS agar we therefore find an excellent selective and advantageous inhibitive medium for the isolation of Eberthella, Salmonella, and Shigella organisms. It readily differentiates these three groups and other nonlactose-fermenting organisms which form opaque, transparent, or translucent uncolored colonies from the brick red-colored coli. Of all the inhibitive, selective media thus far described, SS is one medium which is sufficiently inhibitive to allow a large inoculum and which supports the growth of all intestinal pathogens found in these studies.

The tetrathionate broth has been very useful. In five of the thirty-one positive findings, the organisms were picked up only in the tetrathionate broth. In three of these cases no pathogens were seen in either of the agars used. In two cases, both the SS and MacConkey plates were overgrown by coli. Where there exist too few organisms in the specimen to be recognized on agar, they may multiply in the tetrathionate broth, which inhibits the coli, and then on subculture, from this broth, the pathogens may be more easily isolated. It is also of value in cases where, unfortunately, too large an inoculum is used on solid media and, as a result, discrete colonies are not obtained. Tetrathionate broth was found to support the growth of many strains of Salmonella, Shigella, and Eberthella as listed in Table I.

CONCLUSION

1. SS agar is an excellent culture medium for isolating Eberthella, Salmonella, and Shigella from feces. It gives fine definition of colony, provides for maximum inhibition of coli, and facilitates isolation of intestinal pathogens when a large inoculum is used.

2. If one selective, inhibitive, solid medium were to be chosen for the isolation of all intestinal pathogens, SS agar would seem to be best choice thus far.

3. In conjunction with SS agar, tetrathionate broth is of definite value in the isolation of Eberthella, Salmonella, and a few of the Shigella group.

4. In a small series of positive cases, SS agar was found as efficient or superior to bismuth sulfite agar in the isolation of *E. typhosa* from feces.

We wish to thank Dr. Alfred Angrist, Pathologist of Queens General Hospital, for his many helpful suggestions and kind cooperation.

REFERENCES

1. Liefson, E.: New Culture Media Based on Sodium Desoxycholate for the Isolation of Intestinal Pathogens and for the Enumeration of the Coli Bacilli in Milk and Water, J. Path. & Bact. 40: 581, 1935.
2. Paulson, Moses: The Clinical Use of Desoxycholate and Desoxycholate-Citrate New Culture Media for the Isolation of Intestinal Pathogens, Am. J. M. Sc. 193: 1, 1937.
3. Hardy, A. V., and Watt, J.: Acute Diarrheal Disorders. Am. J. Pub. Health 28: 730, 1938.
4. Hardy, A. V., Watt, T., DeCapito, M., and Kolodny Maxwell, H.: Studies of Acute Diarrheal Diseases. 1. Differential Culture Media, Pub. Health Rep. 54: 287, 1939.
5. Cooper, Merlin L., Furcolow, M. L., Mitchell, A. Graeme, and Cullen, Glenn E.: The Relation of Dysentery to the Acute Diarrhea of Infants and Children, J. Pediat. 15: 172, 1939.
6. Sellers, T. F., Morris, J. F., and Reynolds, M.: Notes on the Application of Bismuth Sulfite Medium to the Isolation of B. Typhosus From Feces, J. Lab. & Clin. Med. 20: 202, 1934.
7. Gunther, C. B., and Tuft, L.: A Comparative Study of Media Employed in the Isolation of Typhoid Bacilli From Feces and Urine, J. Lab. & Clin. Med. 24: 461, 1939.
8. Jones, E. R.: Use of Brilliant Green-Eosin Agar and Sodium Tetrathionate Broth for the Isolation of Organisms of the Typhoid Group, J. Path. & Bact. 42: 455, 1936.
9. Irons, J. V., Bohls, S. W., DeShazo, Thelma, and Hewlett, L. L.: Observations on MacConkeys' and Desoxycholate-Citrate Agars for the Isolation of Dysentery Bacilli, J. Lab. & Clin. Med. 25: 81, 1935.
10. Difco Manual: A New MacConkeys' Agar, 1939.
11. Mayfield, R. Catherine, and Gober, Maud: Comparative Efficiency of Plating Media for the Isolation of Shigella Dysenteriae, Am. J. Pub. Health 31: 363, 1941.
12. Rose, Harry M., and Kolodny, Maxwell H.: Use of SS (Shigella-Salmonella) Agar for the Isolation of Flexner Dysentery Bacilli From the Feces, J. Lab. & Clin. Med. 27: 1081, 1942.
13. Cooper, Merlin L., Keller, Helen M., and Glesne, Lillian R. B.: Bacillary Dysentery in Infants. Evaluation of Three Media, Sodium Desoxycholate-Citrate, SS and MacConkey for the Isolation of Shigella Paradysenteriae From Stools, J. Pediat. 20: 596, 1942.
14. Liefson, E.: New Selenite Enrichment Media for the Isolation of Typhoid and Paratyphoid (Salmonella) Bacilli, Am. J. Hyg. 24: 423, 1936.
15. Mueller, L.: Un Nouveau Milieu d'enrichissement pour la Recherche de Bacilli Typhique et des Paratyphiques, Compt. rend. Soc. de biol. 89: 434, 1923.
16. Kaufmann, F.: Ein Kombiniertes Anreicherungsverfahren fur Typhus- und Paratyphus-Bazillen, Zentralbl. f. Bakt. I Abst. Orig. 119: 148, 1930.
17. Difco Manual: Tetrathionate Broth, 1939.
18. Tabit, F.: A Modification of Wilson and Blair's Bismuth Medium for Both Typhoid and Paratyphoid Bacilli, J. Path. & Bact. 46: 181, 1938.
19. Coleman, Marion B.: The Differentiation and Identification of Bacillary Incitants of Dysentery, Am. J. Pub. Health 30: 39, 1940.

CHEMICAL

SOME SOURCES OF ERROR IN SULFANILAMIDE DETERMINATIONS*

A. H. S. Holbourn, M.A., D.Phil., and R. E. Pattle, B.A.
Oxford, England

THE METHODS commonly adopted for the determination of the concentration of sulfanilamide in blood are liable to errors arising from the neglect of certain variables which strongly influence the values obtained. Until these variables were tracked down, and the errors due to their neglect eliminated, inconsistent results were obtained.

A number of modifications of Marshall's[1] original method have been proposed. But, as will be shown later, these have little or no effect on the errors with which this paper is concerned. These errors arise, for the most part, during the diazotization of the sulfanilamide, and, as the modifications to the original method are mainly concerned with procedures subsequent to diazotization, they do not remove the errors which have already occurred.

As the particular method employed is therefore unimportant, a simple method, based on Marshall's original method, was used wherever possible. The exact procedure is described in the manual of the Evelyn photoelectric colorimeter as follows:

Take 0.2 c.c. whole blood, and add 7.8 c.c. distilled water. Allow to stand for two minutes. Add 2 c.c. 15 per cent toluenesulfonic acid. Mix well and filter. Pipette 4 c.c. of filtrate into a colorimeter tube, add 1 c.c. of 0.025 per cent sodium nitrite solution, mix well and stand for three minutes. Add 5 c.c. of a solution consisting of 1 c.c. dimethyl-α-naphthylamine in 250 c.c. ethyl alcohol. Read the color developed after ten minutes against a blank made up with 4 c.c. of 2.5 per cent toluenesulfonic acid instead of 4 c.c. of filtrate.

Determinations were also made using 10 per cent trichloracetic acid as a protein precipitant instead of toluenesulphonic acid, and 2 per cent trichloracetic acid in the blank instead of 2.5 per cent toluenesulfonic acid.

The sulfanilamide which was the subject of this investigation was added to the distilled water used for hemolyzing the blood in an amount equivalent to a concentration in the blood of 10 mg. per 100 c.c. Human blood, kept fluid by the oxalate crystal method, was used. A filter having a maximum transmission at 520 mμ was used in the colorimeter.

The most important of the numerical results on which the findings below are based are given in Table I. In the column headed "Density" is given 23.8

*From the Nuffield Department of Surgery, University of Oxford, England.
Received for publication, July 28, 1942.

TABLE I

TEMP. OF DIAZOT'N	DENSITY 3 MIN. DIAZOT'N		DENSITY 10 MIN. DIAZOT'N		DENSITY 20 MIN. DIAZOT'N		DENSITY 6 HR. DIAZOT'N	
	T.S.A.	TRICHL.	T.S.A.	TRICHL.	T.S.A.	TRICHL.	T.S.A.	TRICHL.
0° C.	3.5	4.3			9.2	9.7	9.7	
10°	5.7	7.2	9.3	10.0	10.3	10.5		
20°	9.0	9.7	9.7	10.0	10.0	10.0		7.0
40°	9.6	10.0			9.1	10.0		
20° DS	6.5	8.6			0.3	5.4		
20° PS20					10.0	10.0		
20° PS180					3.1	9.0		
20° U	7.4				9.0			

DS means that the diazotization was carried out in sunlight.

PS20 means that after the pink color had developed completely, it was exposed to 20 minutes of sunshine.

PS180 means that after the pink color had developed completely, it was exposed to 8 hours of sunshine.

U means that the nitrite was gently run in and not stirred.

$(2 - \log_{10}G)$, where G is the percentage transmission of the colorimeter tube. The effects of the following variables were studied.

The Effect of Light on the Diazo Reaction.—There was no perceptible difference between diazo reactions which proceeded in the dark and reactions which proceeded five feet away from a 60 watt bulb in a pearl glass shade, under any conditions of temperature or time of diazotization. But the effect of direct sunlight was very pronounced. The sun, in these experiments was always shining from a clear sky, but, unfortunately, this varied considerably in blueness, so that the different densities in the table are not strictly comparable. How important the effect of sunlight can be under certain conditions is shown by the fact that one solution which had been diazotized for twenty minutes in strong sunlight failed to produce any color at all when coupled to dimethyl-α-naphthylamine in the dark. Coupling to N-(1-naphthyl)ethylenediamine, instead of dimethyl-α-naphthylamine after twenty minutes' diazotization in sunlight caused a very faint yellowish color to appear instead of the usual purplish pink color. As one would expect, the effect of sunlight is less pronounced if one diazotizes in sunlight for only three minutes.

Once the diazo compound has been coupled to dimethyl-α-naphthylamine, and the pink color completely developed, twenty minutes' exposure to sunlight has no appreciable effect, although eight hours' exposure changes the pink color to a brownish yellow. According to Bratton and Marshall,[2] the purplish pink ethylenediamine compound is decomposed by sunlight.

Time and Temperature of Diazotization.—All the following results were obtained when the diazotization and the coupling were carried out in artificial light.

For temperatures of diazotization between 0° and 20° C., approximately the same maximum color was obtained on coupling if the diazotization had been carried out long enough. For temperatures between 10° and 20° C., ten minutes was sufficient to produce maximum color when trichloracetic acid was used as the precipitant, and twenty minutes when toluenesulphonic acid was used. A three-minute diazotization did not produce maximum color unless the temperature was raised to 30°. Too high a temperature or too long a time of diazotization produced some destruction of the diazo compound.

The Protein Precipitant.—It was found that with times and temperatures of diazotization which were insufficient to produce maximum color, trichloracetic acid produced somewhat denser color than toluenesulfonic acid. But when the time and temperature were sufficient to diazotize the sulfanilamide fully, the same color density was developed in the presence of either precipitant. With trichloracetic acid, diazotization was more rapid and it was less affected by sunlight, too high a temperature and too long a time of diazotization. Hence, from this point of view, trichloracetic acid is preferable as a precipitant.

The Keeping Qualities of Sulfanilamide in the Presence of Trichloracetic Acid or Toluenesulfonic Acid.—The sulfanilamide content remained constant when the protein free filtrate was kept in the dark at room temperature for fourteen days, with either precipitant.

The Keeping Qualities of the Alcoholic Solution of Dimethyl-α-Naphthylamine.—Perfectly consistent results could be obtained with a solution which had been kept at room temperature for a month. The solution became slightly brownish in this time, but, provided the blank was also made up with this brownish solution, the same readings for density were obtained as with a clear, fresh solution.

The Keeping Qualities of the Pink Dye.—Provided the diazotized sulfanilamide was coupled to the dimethyl-α-naphthylamine in moderate artificial light, full color developed in about ten minutes, for temperatures of coupling between 0° and 20° C. All readings were therefore taken after ten minutes. Even after keeping the pink dye in the dark at room temperature for a week the color was only just measurably darker than after ten minutes.

Improper Mixing of the Nitrite.—If the nitrite was not properly mixed with the protein free filtrate, diminished color was obtained even after a twenty-minute diazotization.

The Dilute Sodium Nitrite Solution.—The dilute nitrite solution (0.025 per cent) was always freshly made up each week from a 1 per cent stock solution. But it was afterwards found that some 0.025 per cent solution of ordinary sodium nitrite which had been kept in the dark for four months gave the same results as freshly made up solution, for concentrations of sulfanilamide equivalent to 10 mg. and also to 30 mg. per 100 c.c. Some dilute nitrite solution which had stood close up againest a window for three months also gave the same results for 10 mg. per 100 c.c. No test was made with this nitrite solution on any stronger solution of sulfanilamide.

There was no difference in the results obtained when using A.R. sodium nitrite, and the results when using ordinary sodium nitrite.

Excess Nitrite and the Effect of Sunlight.—A very large excess of nitrite, say fifty times the usual excess, will inhibit the formation of the pink dye and lead to the formation of a yellowish color instead. Hence the excess nitrite is often removed with urea. When 1 c.c. of 0.25 per cent urea solution was added to a trichloracetic acid and sulfanilamide mixture which had been diazotized for twenty minutes in very strong sunlight, and the whole allowed to stand for one minute before coupling, practically no color was developed. Exactly the same low color density was obtained when the 1 c.c. of urea solution was replaced by 1 c.c. of distilled water. It was also found that when diazotization

had been carried out in the dark, the effects of 1 c.c. of urea solution and 1 c.c. of distilled water were exactly the same.

Use of N-(1-Naphthyl) Ethylenediamine.—Where a twenty-minute diazotization had taken place in very strong sunlight, a pale yellowish color developed on coupling to N-(1-naphthyl)-ethylenediamine instead of the usual purplish pink color. The removal of the excess nitrite did not change this color in any way. Similarly, when the diazotization was carried out in the dark, the removal of the excess nitrite had no effect.

DISCUSSION

Our results differ from those of previous workers in certain particulars of practical importance.

The most important result is the effect of sunlight on the diazo reaction. This does not seem to have been noticed before, possibly because diazotization for only three minutes is the usual practice. Unless the light was strong its effect in this amount of time might escape detection. Nevertheless the effect of light in tropical countries under camp conditions would be of the utmost importance whatever the time of diazotization.

Marshall[3] found that diazotization of sulfanilamide was practically complete in one minute, and no significant difference in color was obtained if the reaction was allowed to proceed for eight minutes. This does not agree with our results. A possible explanation is that Marshall's work may have been carried out at a room temperature considerably higher than usually obtains in Britain.

It is the general practice in diazotization to keep the temperature below 5° C., as most diazo compounds decompose rapidly above this temperature. The naphthylamines and nitroanilines, however, may be diazotized at room temperature without loss of yield. Sulfanilamide also appears to have a diazo compound which is comparatively thermostabile.

As, in our experiments, only free sulfanilamide was present, our results refer strictly only to free sulfanilamide determinations. Methods of determining total sulfanilamide are described by various workers. They are similar to the method given, except that the sample of blood or the protein free filtrate is boiled for about an hour either with the protein precipitant or with hydrochloric acid. This has little effect on subsequent operations, so that our results may be taken as applying without serious modification to determinations of total sulfanilamide.

We found that both trichloracetic acid and toluenesulfonic acid are satisfactory as protein precipitants. Marshall[3] found that both were satisfactory for free sulfanilamide, but that trichloracetic acid was unsatisfactory for total sulfanilamide because, owing to the decomposition taking place during boiling, it did not fully hydrolyze the conjugated compound. Hence Abrahamson[4] boils the filtrate with hydrochloric acid after precipitating the proteins with trichloracetic acid.

Marshall[1] recommends that the dilute nitrite solution should be freshly prepared, and that exceedingly pure nitrite should be used. We find these precautions unnecessary.

Marshall and Litchfield[5] propose certain improvements in the method, viz., removal of excess nitrite with ammonium sulfamate, and buffering of the reaction mixture with acid sodium phosphate. Ratish and Bullowa[6] use urea to remove the excess nitrite. We have found that the moderate excess of nitrite recommended in Marshall's original paper has no measurable effect in any circumstances in which we have made tests. It would certainly be surprising if, when the diazotization is incomplete due to too low a temperature or too short a time of diazotization, the removal of the excess nitrite or of any other of the suggested modifications of the original method completed the diazotization. Hence the time and temperature relationships given in Table I will remain the same.

In their latest method Bratton and Marshall[2] use N-(1-naphthyl)-ethylenediamine as coupling agent. Here they note the effect of light on the completed dye, but not on the diazo compound.

Marshall, Emerson and Cutting[7] find differences of 2-3 per cent between duplicate determinations. The figures given in the table have at least this degree of accuracy.

CONCLUSIONS

The generally recommended precautions have little or no effect in remedying the errors which often arise in the measurement of sulfanilamide. These errors are usually due to the fact that the diazo compound of sulfanilamide is very easily decomposed by light, and to the fact that, unless the room temperature is very high, diazotization is incomplete in three minutes, which is the usual time.

If the diazotization is incomplete, its dependence on time and temperature is very marked. Hence in order to avoid the necessity of accurate control of both the time and the temperature, the diazotization should proceed until it is as nearly complete as possible. Therefore, if trichloracetic acid is used as the protein precipitant, diazotization should proceed for ten minutes at temperatures between 0° and 30° C., but if toluenesulfonic acid is used, twenty minutes is necessary.

SUMMARY

When sulfanilamide is estimated by forming a diazo compound and coupling to dimethyl-α-naphthylamine or N-(1-naphthyl)ethylenediamine, it is recommended that the diazo reaction should proceed at room temperature in moderate artificial light for ten minutes in the presence of 10 per cent trichloracetic acid. With 15 per cent toluenesulfonic acid twenty minutes is necessary. The coupling reaction should also be performed in moderate artificial light.

The effect of sunlight might be very important when making determinations in tropical countries under camp conditions.

We should like to thank Mr. O'Brien and Mr. Higgins of the Radcliffe Infirmary for their valuable criticisms and assistance.

REFERENCES

1. Marshall, E. K., Jr.: Determination of Sulfanilamide in Blood and Urine, Proc. Soc. Exper. Biol. & Med. 36: 422, 1937.
2. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. 128: 537, 1939.

3. Marshall, E. K., Jr.: Determination of Sulfanilamide in Blood and Urine, J. Biol. Chem. 122: 263, 1937.
4. Abrahamson, E. M.: Office Clinical Chemistry, New York, 1940, Oxford University Press, p. 132.
5. Marshall, E. K., Jr., and Litchfield, J. T., Jr.: Determination of Sulfanilamide, Science 88: 85, 1938.
6. Ratish, H. D., and Bullowa, J. G. M.: A Bedside Test for Sulfapyridine, J. Lab. & Clin. Med. 25: 651, 1940.
7. Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: Para-Aminobenzenesulfonamide; Absorption and Excretion: Method of Determination in Urine and Blood, J. A. M. A. 108: 953, 1937.

A SIMPLE CHAMBER FOR ESTIMATION OF SERUM pH WITH A GLASS ELECTRODE*

Alfred H. Free, Ph.D., Cleveland, Ohio

THE development of the glass electrode has led to its widespread use in the determination of pH or hydrogen ion concentration.[1] This electrode has been successfully employed in the measurement of the pH of blood, serum and plasma.[2-4] Recently many manufacturers of laboratory apparatus have placed on the market portable pH meters which are adapted for use with glass electrodes.

In the estimation of pH of blood, serum or plasma it is essential that the measurements be carried out in a closed chamber to prevent loss of CO_2 which will in turn cause changes in pH. Methods of handling blood to prevent CO_2 losses have been adequately discussed.[5, 6] The present report describes a simple chamber which can be used with a glass electrode in the determination of pH of blood serum.

Description of Chamber.—The chamber is made from a four-way glass stopcock in which the four outlets are in the same plane at right angles to one another. The plug of the stopcock has a bore such that any two adjacent outlets can be connected. The outlet tubes have an internal diameter of 1 mm. and 6 mm. external diameter and the plug of the stopcock has a 1 mm. bore. Such stopcocks are available from manufacturers.†

Onto one of the outlet tubes (Outlet II) is fused a larger tube which has a length of 30 mm. and an inner diameter of 13 mm. A larger tube (10 mm. inner diameter and 15 mm. long) is also fused onto the adjacent outlet (Outlet III) which is then bent at right angles as shown in the photograph (Fig. 1).‡

The chamber can conveniently be seated and fastened with rubber bands to a prong of wood which is attached to a small ring stand. The bulb type glass

*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

Received for publication, September 15, 1942.

†Ace Glass Co., Vineland, N. J.; Corning Glass Works, Corning, N. Y.

‡A chamber with these specifications was made for the author by the Dietz Chemical Glass Works, Inc., 1975 E. 65th St., Cleveland, Ohio.

electrode is fitted into the upper chamber of Outlet II by means of a rubber collar placed around the neck of the glass electrode. This collar can conveniently be made from a rubber stopper or a piece of pressure tubing and should be airtight. A piece of 20 gauge syringe needle is placed through the collar to serve as a gas exit. This exit can be readily closed by sticking a small cork on the needle as is shown in the photograph. The calomel half cell is connected to the side chamber by means of a rubber stopper. A small piece of rubber tubing is connected to the horizontal outlet tube (Outlet I). When the glass electrode and calomel cell are connected to the pH meter the instrument is ready for use.

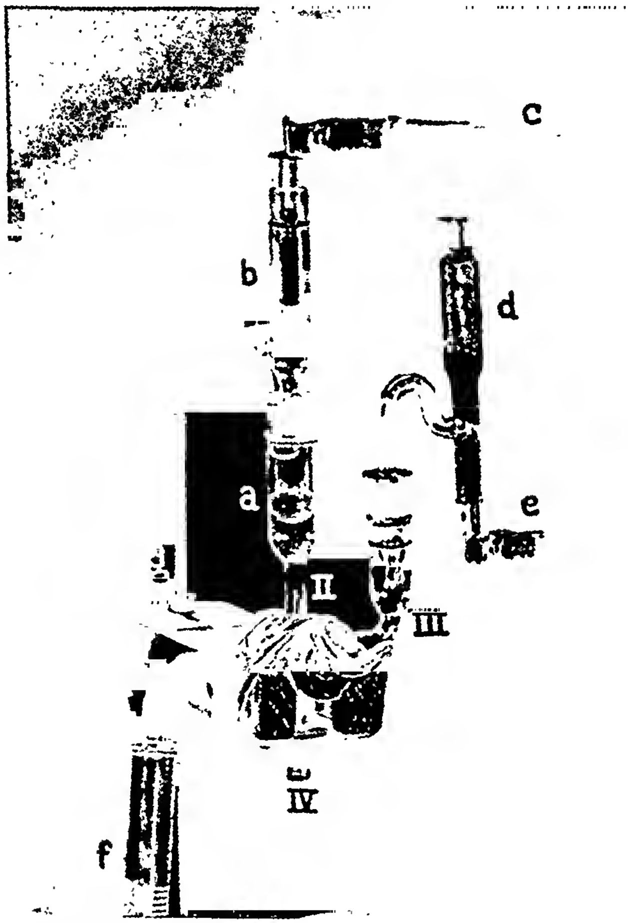


Fig. 1.—Chamber for determination of serum pH. *I*, Stopcock Outlet I for introducing serum sample. *II*, Stopcock Outlet II containing chamber into which bulb type glass electrode is inserted. *III*, Stopcock Outlet III containing chamber for attaching calomel half cell. *IV*, Stopcock Outlet IV which serves to carry away waste KCl and waste mineral oil. *a*, Chamber containing serum ready for measurement. *b*, Glass electrode. The bulb can be seen in the serum chamber. *c*, Lead to pH meter from glass electrode. *d*, Hellige calomel half cell. *e*, Lead from calomel half cell to pH meter. *f*, Syringe in position for introducing sample. *g*, Ring stand with small prolonged wooden block for holding chamber.

The chamber described has been used exclusively with a Hellige pH meter,* a Hellige calomel half cell, and a Hellige bulb type glass electrode (diameter of bulb 10 mm.). However, so far as can be ascertained, there is no reason why the apparatus could not be used with other instruments. It would no doubt be desirable to change the size and shape of the chamber housing the glass electrode if the glass electrode used were of a different design.

*Hellige electrometric pH meter with range control.

Method for pH Determination.—Using a 5 c.c. syringe which contains about 1 c.c. of mineral oil and no air bubbles, 2 c.c. of serum is drawn into the barrel of the syringe. The syringe is inverted so that the oil rises to the top and the needle is removed. The syringe is connected with the rubber tube on Outlet I and the stopcock turned to connect Outlets I and IV. With the syringe still inverted the oil is discharged through Outlet IV into a waste container. By carrying out this operation slowly the stopcock can be turned to connect Outlets I and II as soon as serum reaches the stopcock. The serum is forced into the chamber housing the glass electrode. The stopcock is then turned an eighth of a revolution to the right and a small cork placed over the end of the syringe needle which serves as a gas exit. The stopcock is then turned to connect Outlets II and III and the reading of the pH is made in this position. The stopcock is then turned to connect Outlets III and IV and approximately 2 c.c. of saturated KCL solution allowed to flow through the calomel half cell and out into a waste container. The serum is then allowed to flow out of the chamber by turning the stopcock to connect Outlets I and II and removing the stopper from the needle.

It has been found that if the serum drains completely there is no necessity to rinse out the serum chamber when a series of serum pH determinations are being made and the variation between samples is not more than 0.15 pH. It has furthermore been found that the loss of CO_2 from the serum when it is being introduced into the chamber is negligible. This has been found by introducing a sample and measuring its pH and subsequently withdrawing it through the needle exit while the second sample of the same serum is simultaneously being introduced. By this technique duplicates check to within 0.01 pH as they do when carried out in the conventional manner.

With care mineral oil can be kept out of the chamber and hence off the glass electrode. However, a small amount of oil deliberately introduced into the electrode chamber does not give a value different from a duplicate in which no oil is present.

For serum pH studies a convenient reference buffer is a ⅟₁₅ M. phosphate buffer with pH of 7.38 at 38° C.[7] The buffer is standardized by exactly the same operations used for the serum.

In order to avoid temperature corrections the chamber and electrode system as well as the pH meter are kept at 38° C. This has been accomplished by keeping the whole outfit in a room at 38° C. or by placing the outfit in a large thermostat box maintained at 38° C.

SUMMARY

A simple chamber made from a four-way glass stopcock has been described for use with a glass electrode and pH meter in the determination of serum pH. The technique for measurement of serum pH with the chamber is described.

REFERENCES

1. Dole, M.: The Glass Electrode, New York, 1941, J. Wiley and Sons, Inc.
2. Havard, R. E., and Kerridge, P. T: An Immediate Acid Change in Shed Blood, Biochem. J. 23: 600, 1929.

3. Stadie, W. C., O'Brien, H., and Laug, E. P.: Determination of the pH of Serum at 38° With the Glass Electrode and an Improved Electron Tube Potentiometer, J. Biol. Chem. 91: 243, 1931.
4. Sendroy, J., Jr., Shedlovsky, T., and Belcher, D.: The Validity of Determinations of the pH of Whole Blood at Thirty-Eight Degrees With the Glass Electrode, J. Biol. Chem. 115: 529, 1936.
5. Myers, V. C., and Muntwyler, E.: The Colorimetric Estimation of the Hydrogen Ion Concentration of Blood, J. Biol. Chem. 78: 243-255, 1928.
6. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Baltimore, 1931, Williams and Wilkins Co., Vol. II.
7. Hastings, A. B., and Sendroy, J., Jr.: Studies of Acidosis. XX. The Colorimetric Determination of Blood pH at Body Temperature Without Buffer Standards, J. Biol. Chem. 61: 695, 1924.

NOTE ON THE DETERMINATION OF "CITRIN" IN LEMON PREPARATIONS*

Arthur E. Goldfarb, M.D., Ernest Bueding, M.D., and Philip Karp, B.S.
New York, N. Y.

THE expanding use of lemon extract containing so-called "Vitamin P" or "lemon citrin" in the treatment of psoriasis[1] makes it of interest to determine the "citrin" content of the lemon preparation used.

It was observed that in the determination of "citrin"[2] the reddish-brown color produced by addition of alkali to lemon preparations was increased by heating the samples for two minutes at 100° C. Since carbohydrates produce a brown color on heating with alkali (Moore's reaction), it was investigated whether the presence of sugar interferes with the determination of "citrin," and an attempt was made to eliminate this possible source of error.

The preparation of lemon extract was essentially the same as described by Lorenz and Arnold,[2] except that in one series of experiments the lemon peel was extracted without cooking.

Procedure.—In modification of the method described by Lorenz and Arnold,[2] the following procedure was used for the assay of "citrin" in lemon preparations:

To the solution to be analyzed, an equal volume of alcohol (95 per cent) is added and the mixture filtered. The filtrate is diluted with 50 per cent alcohol so that the color intensity lies within the optimum range of standard eriodictin solutions (lemon peel extract 1:100, lemon juice 1:20, final product being a mixture of both, 1:50). Eight-tenths c.c. of 25 per cent KOH is then added to 9.2 c.c. of this dilution and the color produced is read in the Klett-Summerson colorimeter using filter No. 42, at which time maximum color development occurs.

*From the Departments of Dermatology and Medicine, New York University, College of Medicine, the Dermatological Service of the Third (New York University) Medical Division, and the Medical Service of the Psychiatric Division, Bellevue Hospital, New York City.

Aided by a grant from The California Fruit Grower's Exchange, Inc., Mr. Anthony J. Lorenz—Director of Research.
Received for publication, Sept. 18, 1942.

Determinations of eriodictin solutions (A52) of known concentration showed that the optimum range falls between 0.04 and 0.2 mg. eriodictin per cubic centimeter in which a straight-line relationship is observed between concentration and colorimeter reading.

Effect of Glucose on Colorimetric Determination of "Citrin."—It was found that both lemon juice and lemon peel extract contain about 1 per cent of fermentable reducing monosaccharides. While this amount of glucose (0.05 per cent in a dilution of 1:20 of lemon juice) when added to standard eriodictin solutions does not produce any color reaction, a measurable color is produced with the above procedure after heating for two minutes. On the other hand, in all lemon preparations the apparent "citrin" value was higher after heating for two minutes than in the unheated sample. This indicates that on heating the samples, glucose and possibly other substances interfere with the "citrin" determination and, therefore, give higher values than correspond to the true "citrin" content of the lemon preparation.

Effect of Cooking on "Citrin" Content of Lemon Peel Extract.—The colorimetric determination of the cooked extract gives significantly lower values than the uncooked, indicating that some "citrin" is destroyed on cooking. The uncooked extract was obtained by allowing the ground peel to stand in cold water at room temperature for twenty-four hours or more.

"Citrin" Content of Lemon Juice.—A measurable quantity of "citrin" can be found in lemon juice; its concentration is about one-sixth of that contained in the uncooked peel extract. Whether the compound found in lemon juice has biological activity is yet to be determined.

When lemon peel extract and juice whose "citrin" contents have been determined separately are combined, the determination of this mixture yields the "citrin" value calculated from the figures for each component.

SUMMARY

1. A modification of the method of Lorenz and Arnold for the determination of "citrin" in lemon preparations is described, and the interference of glucose is eliminated by avoiding heating of the solutions after the addition of alkali.

2. A more efficient extraction of "citrin" from lemon peel is obtained by extracting in the cold instead of by cooking.

3. Lemon juice contains about one-sixth the amount of "citrin" found in lemon peel extract.

REFERENCES

1. Goldfarb, A. E.: Treatment of Psoriasis With Lemon Citrin (Vitamin P) With Lemonade and Ascorbic Acid, Arch. Dermat. & Syph. 43: 356, 1941.
2. Lorenz, A. J., and Arnold, L. J.: Preparation and Estimation of Crude Citrin Solutions (Vitamin P) From Lemons, Food Research 6: 151, 1941.

DETERMINATION OF URIC ACID IN WHOLE BLOOD AND SERUM*

J. W. MULL, PH.D., CLEVELAND, OHIO

A STUDY of the uric acid content of the blood was undertaken with the threefold purpose of selecting a practical method for clinical determinations using a photoelectric colorimeter, establishing the normal range for uric acid by this method, and ascertaining the relation between the uric acid content of whole blood and of serum.

The colorimetric method of Benedict and Behre[1] on the silver chloride filtrate, while a most satisfactory method for a visual colorimeter, is unsuited to the photoelectric colorimeter since it must be read while hot. The method proposed by Brown[2] depends on exact timing, and with the great sensitivity of the photoelectric instrument the changes are so rapid as to preclude reading checks. That of Newton,[3] in addition to rather elaborate reagents, did not give a curve that could be duplicated with successive determinations on the standard. Although Folin's method,[4] permits the development of the color at room temperature, it requires too much time in the preparation of reagents to be available for clinical use. We therefore turned back to the simple, short, but direct method of Benedict,[5] to which we applied the uricase treatment of Blauch and Koch.[6] Since uricase destroys uric acid, the color developed after the addition of uricase represents impurities. This value subtracted from the untreated findings gives the true uric acid content.

METHOD

To separate 1 c.c. samples of whole blood and serum was added 25 mg. uricase powder. The tubes were then capped and allowed to incubate for two hours in a water bath held between 40 and 48 degrees C. Following incubation, the specimens, together with fresh 1 c.c. samples of the same whole blood and serum, were precipitated with 1 c.c. 10 per cent sodium tungstate, followed by 8 c.c. $\frac{1}{12}$ normal sulfuric acid. After thorough stirring, especially with the incubated tubes, they were allowed to stand ten minutes, then centrifuged.

A 2 c.c. aliquot of the clear supernatant fluid was pipetted into tubes and diluted to 10 c.c. with distilled water. To each was added 4 c.c. 5 per cent sodium cyanide, followed by 1 c.c. arseno-phosphotungstic acid. The tubes were inverted once and placed immediately in a boiling water bath for three minutes, removed from the bath and allowed to stand about one minute at room temperature, one minute in cold water, and were read after a total of three minutes and within the next five. A 650 mμ filter was used, reading on a logarithmic scale. Too rapid cooling or delay in reading usually resulted in clouding.

*From the laboratory of the Maternity Hospital and the Department of Biochemistry, Western Reserve University School of Medicine.

Received for publication, Sept. 21, 1942.

The 5 per cent sodium cyanide was made up in water containing 1 c.c. concentrated ammonium hydroxide per 500 c.c. The arseno-phosphotungstic acid was prepared by dissolving 100 Gm. sodium tungstate in 600 c.c. water, adding 50 Gm. pure arsenic pentoxide, 25 c.c. syrupy phosphoric acid (85 per cent H_3PO_4), and 20 c.c. hydrochloric acid in a liter flask. The mixture was boiled 20 minutes, cooled, and diluted to 1 L. This reagent keeps indefinitely.

The standard was prepared as recommended by Benedict and Hitchcock,[7] and contained 0.2 mg. per 1 c.c. This keeps at room temperature about one month if tightly stoppered and only opened infrequently. For use, 1 c.c. was diluted to 100 c.c. Samples of 1, 2, 4, and 5 c.c. of this diluted standard, corresponding to 1, 2, 4, and 5 mg. per 100 c.c. of blood, were used to establish a curve, plotting the electrophotometer readings against the concentrations. This curve was found to alter slightly with successive daily readings, and it was found necessary to repeat it every third day, using freshly diluted standard. The points on these curves invariably plotted in a straight line, the alteration being due to change in the 5 per cent sodium cyanide solution. Neither using different brand preparations of cyanide nor the addition of urea prevented this alteration, which was quite probably due to change in the ammonia concentration.

RESULTS

Employing the method as outlined, we examined fifty-one male medical students, twenty-two normal young women, and thirty-seven normal women in the last trimester of pregnancy. A complete summary of the findings on the male group is given in Table I. The means for the whole blood and serum uric

TABLE I

URIC ACID DETERMINATIONS ON 51 HEALTHY YOUNG MALES

(Values in Milligrams per 100 c.c. Blood or Serum)

	MEAN	S. D.	RANGE	EXCEPTIONS
Whole blood	3.59	0.77	2.05- 5.13	2 low, 1 high
Whole blood plus uricase	1.67	0.51	0.65- 2.67	0 low, 2 high
True uric acid	1.92	0.58	0.76- 3.02	1 low, 1 high
Serum	4.30	0.92	2.46- 6.14	2 low, 0 high
Serum plus uricase	1.07	0.42	0.23- 1.91	0 low, 1 high
True uric acid	3.23	0.60	2.03- 4.43	2 low, 0 high
Hemoglobin	15.51	1.23	13.05-17.97	2 low, 0 high

The range is determined by adding and subtracting 2 times the standard deviation from the mean; theoretically it includes 95 per cent of all determinations.

Correlation of the direct uric acid values of the whole blood and serum is plus 0.857.

Correlation of the true uric acid values of the whole blood and serum is plus 0.89.

Correlation of the direct whole blood uric acid values and the hemoglobin is minus 0.139.

acid, each of which is well over four times the standard deviation (S. D.), are significant, although the values are somewhat closer than had been anticipated. Moreover 95 per cent of the determinations made fall within the range according to statistical theory. There is some question, however, as to the significance of the means of the whole blood with uricase, the true uric acid of the whole blood, and the serum plus uricase, which are only about three times the standard deviation. The hemoglobin values are definitely significant, as the mean is well over twelve times the standard deviation. In the indirect method the correlation

figure of plus 0.89 between the amount of true uric acid in the whole blood and the amount in the serum indicates a definite relationship between the two. There is likewise a relation between the uric acid of the whole blood and of the serum in the direct method, with a correlation figure of plus 0.857. On the other hand, there is no relation between the uric acid of the whole blood and the hemoglobin, with a correlation value of minus 0.139.

TABLE II

COMBINED URIC ACID FINDINGS ON 51 MALES AND 22 FEMALES

(Values in Milligrams per 100 c.c. Blood or Serum)

	MEAN	S. D.	RANGE	EXCEPTIONS
Whole blood	3.53	0.707	2.11-4.91	2 low, 1 high
True uric acid	1.77	0.565	0.64-2.90	0 low, 4 high
Serum	4.14	0.866	2.41-5.87	2 low, 0 high
Serum plus uricase	1.19	0.393		
True uric acid	2.95	0.734	1.48-4.42	1 low, 1 high

Correlation of direct serum uric acid and the serum plus uricase values is plus 0.333.
Correlation of direct whole blood and true uric acid values is plus 0.727.
Correlation of direct serum and true serum uric acid values is plus 0.951.

A very brief summary of the combined findings of the young men and women are given in Table II. These means, from three to five times their respective standard deviations, are all significant. The ranges, while somewhat more restricted than those for the men alone, have no more exceptions than were found in Table I.

The most interesting observation is the correlation found between the uric acid as determined in the whole blood and the true uric acid, plus 0.951. This would indicate that, although there seems to be no relation between the direct determinations of uric acid and the impurities, as for example, the correlation value of plus 0.333 between serum and serum plus uricase, there is a definite relation between the direct determinations and the true uric acid. One is therefore justified in using the direct method on either whole blood or serum, provided only that the results are compared with the normal range established by this method.

TABLE III

DETERMINATIONS ON 37 WOMEN IN THE LAST TRIMESTER OF PREGNANCY

(Values in Milligrams per 100 c.c.—Volumes in Per Cent)

	MEAN	S. D.	RANGE	EXCEPTIONS
Uric acid in whole blood	3.21	0.648	1.90- 4.50	1 low, 3 high
True uric acid	1.19	0.504	0.18- 2.20	1 low, 1 high
Uric acid in serum	3.59	0.94	1.71- 5.47	0 low, 3 high
True uric acid	2.24	0.761	0.72- 3.76	0 low, 2 high
Hemoglobin	10.53	1.28	7.97-13.09	0 low, 0 high
Red cell volume	32.60	3.946	24.70-40.50	0 low, 2 high

Correlation of direct uric acid values in the whole blood and the red cell volume is minus 0.109.
Correlation of the direct uric acid values in the serum and the red cell volume is minus 0.161.
Correlation of the direct and true uric acid values of the whole blood is plus 0.89.
Correlation of the direct and true uric acid values of the serum is plus 0.97.

Table III summarizes the findings on pregnant women in the last trimester. Here again the whole blood and serum uric acid means are four to five times

their respective standard deviations, and therefore significant. While those of the true uric acid by the indirect method are of less definite significance, their range includes the theoretical proportion of the determinations. Both the hemoglobin and red cell volume means are definitely significant. There is no correlation between the uric acid either of the whole blood or of the serum with the red cell volumes. There is, however, excellent correlation between the whole blood and true uric acid, and between the serum and true uric acid, with values of plus 0.89 and plus 0.97 respectively.

In view of these findings, particularly that of the apparent correlation between the whole blood and serum uric acids, it would appear that uric acid determinations on whole blood are fully justified. The complete lack of correlation between whole blood uric acid and either hemoglobin or red cell volume, and also the absence of correlation between the serum uric acid and the hemoglobin or cell volume confirms the belief that it is unnecessary to use serum for uric acid.

TABLE IV

URIC ACID DETERMINATIONS ON INDIVIDUAL PREGNANCY CASES
(Values in Milligrams per 100 c.c. Blood or Serum)

	DATE	DIRECT WHOLE BLOOD	TRUE WHOLE BLOOD	DIRECT SERUM	TRUE SERUM	
D. M.	4/22/42	6.75	3.35	9.30	6.60	Near term
	4/23/42	5.40	2.40	7.85	5.45	
	5/ 6/42	3.30	1.40	5.45	3.95	
W. S.	9/ 2/42	9.60	7.3			Aborted at about 8 weeks
	9/ 8/42	3.20	2.2			

Table IV is included to illustrate further the correlation between the direct determinations of uric acid in whole blood or serum and the pure uric acid values found by the uricase method, even when the uric acid retention is above normal. The first case, D. M., was a diabetic near term; the second, W. S., had suffered a spontaneous abortion at about eight weeks, due to an Rh reaction, with a resulting retention that raised her blood urea nitrogen to 115 mg. per 100 c.c. In both cases the ratio of the values obtained with Benedict's direct method to the true uric acid values during retention is proportional, within the limits of correlation, to the ratio of the same determinations in the normal range. This further justifies the employment of the direct method.

SUMMARY

The direct method of Benedict was found most suitable for determining uric acid with the photoelectric colorimeter.

Either whole blood or serum may be used since there is a direct correlation between the findings.

There is also excellent correlation between the direct whole blood or serum determinations and the true uric acid findings made with the aid of uricase.

Ranges have been established for uric acid by both the Benedict direct method and the same method, after correction with the aid of uricase, for both

whole blood and serum, in males, males and females, and in normal pregnant women.

REFERENCES

1. Benedict, S. R., and Behre, J. A.: The Analysis of Whole Blood. III. Determination and Distribution of Uric Acid, J. Biol. Chem. 92: 161, 1931.
2. Brown, H.: The Determination of Uric Acid in Blood, J. Biol. Chem. 68: 123, 1926.
3. Newton, Eleanor B.: A Chromogenic Tungstate and Its Use in the Determination of the Uric Acid of Blood, J. Biol. Chem. 120: 315, 1937.
4. Folin, O.: Standardized Methods for the Determination of Uric Acid in Unlaked Blood and Urine, J. Biol. Chem. 101: 111, 1933.
5. Benedict, S. R.: The Determination of Uric Acid in Blood, J. Biol. Chem. 51: 187, 1922. Benedict, S. R.: The Determination of Uric Acid, J. Biol. Chem. 51: 233, 1922.
6. Blauch, Mary B., and Koch, F. C.: A New Method for the Determination of Uric Acid in Blood, With Uricase, J. Biol. Chem. 130: 433, 1939.
7. Benedict, S. R., and Hitchcock, E. H.: On the Colorimetric Estimation of Uric Acid in Urine, J. Biol. Chem. 20: 619, 1914-15.

QUANTITATIVE DETERMINATION OF BILE PIGMENT IN THE URINE*

A Comparative Study and Description of a Rapid, Direct, Photocolorimetric Method

Karl Singer, M.D., and Rosa Kubin, Ph.D., Boston, Mass.

DURING the last few years several methods for the quantitative determination of bile pigment in the urine have been published. Although such determinations may at present appear to be of but limited diagnostic value in the clinical study of liver diseases, they are of great importance for investigations of the hemoglobin and bile pigment metabolism in patients and experimental animals (renal biliary fistula dog[1]). Obviously, any quantitative determination of urinary bile pigment excretion should attempt to include all bile pigment compounds which could possibly be present. According to the view of Lemberg,[2] the primary bile pigment (not linked to globin) which develops in the course of hemoglobin catabolism, is not bilirubin but biliverdin (dehydrobilirubin). This substance, however, is immediately and completely reduced to bilirubin in the body, at least in man and dogs. In certain other species (frogs, birds) this reduction does not occur, and the color of the bile therefore is not brown but bluish green.[3] Under certain pathological conditions, e.g., in starvation (Thierfelder[4]), the reduction of biliverdin may also be incomplete and then the bile is composed of a mixture of both pigments. Furthermore, when bilirubin containing urine is exposed to light and air, a rapid, secondary reoxidation to biliverdin and sometimes to even higher oxidation products (choletelin) occurs. Thus, determinations of bilirubin only, are apt to give an inaccurate picture of bile pigment excretion.

At the present time the available quantitative methods for urinary bile pigments can be classified in two different ways. (1) according to the type of

*From the Department of Clinical Pathology, Middlesex University, Waltham, Mass.
Received for publication, Sept. 23, 1942.

chemical reaction used for the determination; (2) in regard to the preceding preparation of the urine for the performance of this chemical reaction, i. e., either separation of the bile pigments by precipitation, or direct determination in the urine without any preparation. As biliverdin cannot be diazotized, methods employing the van den Bergh reaction determine bilirubin only (Goodson and Sheard,[5] Scott,[6] Jendrassik and Czike,[7] and others[8]). If oxidation reagents are used, biliverdin besides bilirubin is included in the result (Hooper and Whipple,[9] Rous and McMaster,[10] and Knutti, Hawkins and Whipple[11 a, b]).

As the varying color of the natural urinary bile pigments (uroerythrin, urochrome, urobilin, etc.) interferes with the color obtained from the interaction between the bile pigments and the added reagents, most methods first separate the bile pigment compounds. Various precipitants are employed: calcium chloride (Hooper and Whipple[9]), barium salts (Scott[6]), trichloracetic acid (Kerpolla[12]). Such a precipitation is time consuming and increases considerably the inaccuracy inherent to any chemical performance. Recently Goodson and Sheard[5] found a way to avoid precipitation by excluding the disturbing natural pigments by means of an appropriate filter in the photoelectric colorimeter. This elegant method, therefore, permits a direct determination. Goodson and Sheard,[5] however, used the diazo reaction in their studies. Since we believe, for reasons which have already been broached, that oxidation methods are to be preferred in determining urinary bile pigments, we have worked out a direct photelometric method. This method is presented in this paper together with a comparative study of the other types of methods available for quantitative determination of urinary bile pigment compounds.

METHOD

Hammarsten's[13] reaction was used for the oxidation of the bile pigments. This reaction consists in the development of a persistent greenish-bluish color, when a mixture of hydrochloric and nitric acid in ethyl alcohol is added to fluids containing bilirubin or biliverdin. The reaction is quite analogous to the Gmelin test, but has the advantage that the oxidation remains arrested at a certain level and does not rapidly proceed further to colorless compounds, as is the case in the Gmelin test. This makes Hammarsten's[13] procedure suitable for colorimetric purposes.

Solutions Required.—(1) Hammarsten's reagent: the stock solution is prepared by mixing 19 parts of a 25 volume per cent hydrochloric acid with 1 part of a 25 volume per cent nitric acid. After twenty-four hours the stock solution is ready for use and then keeps indefinitely. For each test, 1 c.c. of the stock solution is diluted with 4 c.c. of absolute ethyl alcohol. (2) Blank solution: 7.5 c.c. of concentrated hydrochloric acid are diluted with water to make 1 liter.

Procedure.—The pH of the urine should first be tested with nitrazine paper. If the pH is higher than 6, a few drops of glacial acetic acid should be added to obtain an acid urine. Two test tubes or two graduated 10 c.c. cylinders are designated as "blank" and "test" respectively. Into each tube 1 to 2 c.c. of the centrifuged or filtered urine are then poured and 2 c.c. of 95 per cent alcohol are added, the contents are then well mixed by rotating the tubes. Then to the "blank" tube 2 c.c. of the blank reagent are added slowly, and to the "test" tube 2 c.c. of the diluted Hammarsten reagent. Both solutions, after

mixing again, are further diluted up to 10 c.c. by means of alcohol. The bluish-greenish color develops readily in the "test" tube and reaches its maximal intensity after two to five minutes. In the "blank," cloudiness caused by the precipitation of salts by the alcohol, may sometimes be noticeable. In this case the solution is filtered until completely clear. Occasionally it may be difficult to obtain a clear filtrate, in which case the "blank" may be prepared with distilled water instead of alcohol, without influencing the colorimetric evaluation.

Fisher's photoelectrocolorimeter (microset) was available in our laboratory. The red filter (650 $m\mu$) was used. Although no special spectrophotometric studies could be performed, our results confirm the findings of Goodson and Sheard[5] that the disturbing influence of the various urinary pigments can be eliminated by the use of an appropriate filter if the diluted, untreated and acidified urine is employed as a standard for the photelometric comparison. The "zero-point" was determined with ethyl alcohol. Then the "blank" was read and the figure obtained was subtracted from the figure of the "test" solution.

Standardization.—From a stock solution of 20 mg. of bilirubin (Pfannenstiel) in 100 c.c. chloroform, convenient series dilutions were prepared and the oxidation tests performed. The photelometric readings were plotted in a calibration curve on semilogarithmic paper. Like the "diazoreaction" curve, the "oxidationreaction" curve obtained, is not a strictly straight line. The most reliable readings are obtained for a concentration of 0.15 to 0.4 mg. bile pigment per c.c. of urine. If the concentration in a urinary sample should be found out of this range, adaptation to the test by adequate increase or decrease of the amount of urine used is recommended.

COMPARATIVE STUDY

Urine samples from four dogs with renal biliary fistulas* and from four patients with extrahepatic obstructive jaundice were used for the comparison of the above described method with three other types of determination of biliary pigment. These other methods were (1) the direct diazo method of Goodson and Sheard,[5] in which caffeine sodium benzoate is employed as an accelerator in the development of the color reaction, (2) the method of Whipple and his associates,[11] employing calcium chloride for precipitation and nitric acid for oxidation, (3) Scott's[6] method using barium salts for precipitation and the diazo reaction for the determination. The results obtained with the four methods can be seen in Table I; each value therein is the arithmetic average of two parallel determinations.

In most of the experiments the highest values found were with the new direct oxidation method. In order to have a basis for a comparison of the different methods the results of the direct oxidation are assumed to equal 100 per cent and the other values are computed on this assumption (Table II).

DISCUSSION

1. *Comparison of the Two Direct Methods.*—(New oxidation method and diazo method of Goodson and Sheard,[5] Columns 3 and 4 of Tables I and II).

*We want to express our gratitude to Dr. Louis Bergmann and Dr. Leo Weisz for their cooperation in performing the operations.

Performance of the direct oxidation test requires only one-third of the time needed for the diazo test. Both methods give almost identical results with the urines of jaundiced patients (No. 11 to 14). In these experiments only freshly voided specimens were used. With fresh dog urine also, the same results were obtained (No. 8). In all the other animal experiments a twenty-four-hour urine was collected in a brown flask containing chloroform as a preservative,[11] the animal being in the metabolic cage. The diazo values, measuring bilirubin only, were always lower in these specimens, sometimes quite considerably. This is best explained as a result of the secondary reoxidation of the excreted bilirubin.

That bilirubin, when exposed to light and air, may rather rapidly disappear from a urinary specimen, has been repeatedly observed. (Rabinowitch,[14] Sivo and Forrai,[15] Scott[6]). Other factors, like temperature and ultraviolet rays, may also be of contributing importance (Peterman and Cooley[16]). The disappearance of bilirubin is caused mainly by reoxidation to compounds no longer giving the diazo reaction. The higher values of the oxidation method demonstrate clearly the superiority of this type of determination. However, the lower values with the diazo test may sometimes also be due to other causes. During the course of our experiments with the renal biliary fistula dogs almost daily determinations of the bile pigment output were performed using the method of Goodson and Sheard.[5] Immediately after operation the proper reddish-purplish color of diazobilirubin was obtained; later on, however, varying from a few days to a few weeks postoperatively, the diazoreagent, when added to the urine, produced only a brown or even a green color, unrelated to the pH of the specimen. Simultaneously with this phenomenon the urine began to contain great amounts of indican (highly positive Jolles and Obermeyer tests). That indican or related substances may interfere with the van den Bergh reaction in the plasma has been noted by previous investigators. Andrewes,[17] Harrison and Bromfield,[18] and also Becher[19] mention particularly the "buff or brown diazoreaction" in uremia where indican accumulates in the blood. The cause of the considerable indicanuria in our renal biliary fistula dogs was presumably the complete absence of bile from the intestine; bile was not administered to these animals because it would have interfered with a simultaneous investigation using these same dogs.

Two further questions deserve a brief discussion. (1) Does the Hammarsten reaction used in the direct oxidation test determine bile pigment compounds only? Hans Fischer and Adler[20] studied the different phases of the similar Gmelin test and found that other substances like malachite green, crystal violet, dimethylaminoparabenzol, etc., may give similar color compounds. All these substances are, however, unlikely to be present in any pathological urine. Indican, according to Naumann,[21] gives a very slowly developing bluish color with trichloracetic acid and Ferric chloride (Fouchet's reagent). We found in numerous urines not containing any bile pigment that indican, normal urinary pigments, and also stercobilinogen, prepared from feces, if added to the urine, did not give a positive Hammarsten test. Indican alone gives a brown color with the diazo reagent. (2) The second problem is whether the direct oxidation method determines all the excreted bile pigments or whether a loss occurs due to superoxidation to choletelin or colorless compounds. In order to answer this question dog

TABLE I

COMPARISON OF 4 METHODS FOR URINARY BILE PIGMENTS

COLUMN	1	2	3	4	5	6
			BILIRUBIN IN MILLIGRAMS PER 100 C.C. OF URINE			
			DIRECT METHODS		PRECIPITATION METHODS	
					CA CHLORIDE	BA ACETATE
	NO.	TYPE OF URINE	OXIDATION METHOD	DIAZO METHOD GOODSON-SHEARD	OXIDATION METHOD WHIPPLE AND ASSOC.	DIAZO METHOD SCOTT
	1	Dog I	58.0	49.8	47.2	36.3
	2	Dog 1	59.5	54.2	42.6	39.8
	3	Dog 1	63.5	33.9	53.8	35.9
	4	Dog 1	45.0	31.2	47.0	27.5
	5	Dog 2	180.0	160.0	145.0	107.5
	6	Dog 2	155.0	137.5	140.0	131.3
	7	Dog 2	212.5	188.5	195.0	169.8
	8	Dog 3	66.9	67.3	not performed	
	9	Dog 3	125.0	80.0	100.5	73.3
	10	Dog 4	35.5	30.0	31.6	23.0
	11	Patient I	34.0	34.8	39.1	28.8
	12	Patient II	91.1	93.5	73.3	57.0
	13	Patient III	23.2	23.2	16.6	not performed
	14	Patient IV	17.4	17.4	not performed	

TABLE II

DIFFERENCES IN THE FOUR METHODS EXPRESSED IN PER CENT OF DIRECT OXIDATION METHOD

COLUMN	1	2	3	4		5		6	
			BILIRUBIN IN MILLIGRAMS PER 100 C.C. OF URINE						
						PRECIPITATION METHODS			
						CA CHLORIDE		BA ACETATE	
	NO.	TYPE OF URINE	DIRECT OXIDATION METHOD	DIRECT DIAZO METHOD GOODSON-SHEARD		OXIDATION METHOD WHIPPLE AND ASSOC.		DIAZO METHOD SCOTT	
				%	DIFFERENCE	%	DIFFERENCE	%	DIFFERENCE
	1	Dog 1	100%	85.9	−14.1	81.4	−18.6	62.6	−37.4
	2	Dog 1	100%	91.1	− 8.9	71.6	−28.4	66.9	−33.1
	3	Dog 1	100%	53.3	−46.7	84.7	−15.3	56.5	−43.5
	4	Dog 1	100%	69.3	−30.7	104.4	+4.4	61.1	−38.9
	5	Dog 2	100%	88.9	−11.1	80.6	−19.4	59.7	−40.3
	6	Dog 2	100%	88.7	−11.3	90.3	− 9.7	84.7	−15.3
	7	Dog 2	100%	88.7	−11.3	91.8	− 8.2	79.9	−20.1
	8	Dog 3	100%	100.5	+0.5	not performed			
	9	Dog 3	100%	64.0	−36.0	80.4	−19.6	58.6	−41.4
	10	Dog 4	100%	84.5	−15.5	89.0	−11.0	64.8	−35.1
	11	Patient I	100%	102.4	+2.4	115.0	+15.0	84.7	−15.3
	12	Patient II	100%	99.4	−0.6	77.9	−22.1	60.6	−39.4
	13	Patient III	100%	100.0	0.0	71.6	−28.4	not performed	
	14	Patient IV	100%	100.0	0.0	not performed			

bile was taken from the gall bladder and the bile pigment content was immediately determined with both direct methods. Then the bile was protected against light and air by placing it in a dark bottle and adding mineral oil. In the fresh dog bile no biliverdin was present, as can be seen from the identical values with both the diazo and the oxidation tests (Table III). The values of the protected and also of the unprotected bile samples were then compared after twenty-four hours. Similar experiments were performed with urines. The results are compiled in Table IV.

TABLE III

EFFECT OF LIGHT AND AIR ON FLUIDS CONTAINING BILE PIGMENT

NO.	TYPE OF SAMPLE	IMMEDIATE DIRECT DETERMINATION		BILIRUBIN IN MILLIGRAMS PER 100 C.C.							
				AFTER 20 TO 24 HOURS							
				PROTECTED FROM LIGHT				EXPOSED TO LIGHT			
		OXIDATION	DIAZO	OXIDATION		DIAZO		OXIDATION		DIAZO	
		MG. IN 100 C.C.	MG. IN 100 C.C.	MG. IN 100 C.C.	LOSS %	MG. IN 100 C.C.	LOSS %	MG. IN 100 C.C.	LOSS %	MG. IN 100 C.C.	LOSS %
1	Dog bile	1400	1408	1400	0	1236	12.2	1150	17.9	1134	19.5
2	Dog bile	860	880	860	0	360	59.1	495	42.5	430	51.1
3	Dog bile	1488	1488	1480	0.5	1280	13.9	1140	23.4	1060	28.6
4	Dog urine	68	58	67	1.5	56	3.4	67	1.5	51	12.1
5	Dog urine	157.5	137.5	157.5	0	120	12.7	131.3	16.6	100.5	27.0
6	Dog urine	212.5	188.2	177.5	16.5	147.5	21.6	157.5	25.9	122.5	34.9
7	Dog urine*	520	504	500	3.9	400	20.7	120	77	300	40.5
8	Dog urine*	330	335	320	3.0	234	30.2	216	34.6	148	55.8

*The high values occurred after injection of phenylhydrazine chloride.

TABLE IV

QUANTITATIVE RECOVERY OF BILIRUBIN ADDED TO URINE

SAMPLE NO.	MILLIGRAMS OF BILIRUBIN PER C.C.				PERCENTAGE RECOVERED
	IN URINE	IN BILIRUBIN SOLUTION	IN URINE PLUS BILIRUBIN		
			FOUND	ANTICIPATED	
1	none	0.20	0.190	0.200	95.0
2	none	0.26	0.250	0.260	96.1
3	none	0.37	0.380	0.370	102.7
4	none	0.55	0.560	0.550	101.8
5	none	0.89	0.810	0.890	91.0
6	0.135	0.17	0.273	0.305	89.5
7	0.140	0.17	0.279	0.310	90.0
8	0.320	0.17	0.510	0.490	104.1
9	0.137	0.30	0.430	0.437	98.4
10	0.770	0.21	0.950	0.980	96.9

Average recovery 96.6 per cent; range 89.5 to 104.1 per cent.

With one exception (No. 6, Table III) the value obtained with the oxidation test remained constant in the protected sample whereas all the other values decreased considerably. From these results it may be concluded that the oxidation test in samples adequately protected against superoxidation gives probably the most reliable picture of actual bile pigment excretion, although the possibility of a loss caused by superoxidation must always be kept in mind when balance studies of the pigment metabolism are performed.

2. *Comparison of the Two Oxidation Methods.*—(New direct method and precipitation method of Whipple and associates,[11] Columns 3 and 5, Tables I and II). The values estimated with the precipitation method showed deviations from those with the direct test covering a range from –28.4 per cent to +15.0 per cent, with an average deviation of –13.4 per cent. The standard deviation σ is 18.5 per cent. Most of the values with the precipitation method are lower than those with the simpler direct test. This is partly due to the loss to be expected from the more complicated chemical procedure, and perhaps partly caused by the difficulties in achieving the correct degree of oxidation of the bile pigments. The method of Whipple and his associates[11] requires an experienced worker as the amount of their oxidation reagent added to the urine

must be carefully adapted to the constantly varying concentration of pigment present, which factor may result in an insufficient or too efficient oxidation of the bile pigment compounds. These dangers are eliminated in the direct photelometric test. Peterman and Cooley[16] studied the various conditions of significance in obtaining maximal oxidation of bile pigments and recommended hydrogen peroxide as the most suitable oxidation reagent. Malloy and Evelyn,[22] following their suggestion, described a photo-electric method using hydrogen peroxide for determination of bile pigment in bile, feces and meconium. In our experience oxidation with hydrogen peroxide does not give satisfactory results with urinary bile pigment. Peterman and Cooley,[16] and Malloy and Evelyn[22] also expressed the opinion that the green color of fluids containing various bile pigments is not due to the presence of biliverdin. According to these authors biliverdin does not exist as a chemical entity but represents only a mixture of unoxidized yellow bilirubin and blue bilicyanin. This is in sharp contradiction to Lemberg,[2, 3] who defines biliverdin as a bile pigment compound having two hydrogen atoms less than bilirubin and an open α but an intact γ methene bridge. Both statements are most likely only at variance as far as the terminology is concerned. Biliverdin is a definite chemical substance but the green color of fluids containing bile pigment may also sometimes be caused by the presence of a mixture of unoxidized and oxidized pigments. In order to demonstrate that the direct oxidation test which yields a bluish-greenish color, does not leave any bilirubin undetermined, quantitative recovery experiments of bilirubin added to urinary samples were performed. The bilirubin was dissolved in chloroform and then added to urines with and without natural bile pigment. When performing the test, no technical difficulties were encountered, as chloroform in small amounts is readily soluble in alcohol, which is the main fluid medium of the test.

The recovery of bilirubin is as complete as can be expected from such an experiment. The range of the results was 89.5 per cent to 104.1 per cent and the average recovery 96.6 per cent. These values are almost identical with those found by Goodson and Sheard[5] in similar recovery experiments with their direct diazo method.

3. *Comparison of the Two Diazo Methods.*—(Direct method of Goodson and Sheard[5] and precipitation method of Scott,[6] Columns 4 and 6 of Tables I and II). If bilirubin only is to be determined in the urine, the direct oxidation method is doubtless the method of choice. The direct method is not only simpler to perform, but combines, as can be seen from the tables, this advantage with a much higher degree of accuracy.

CONCLUSIONS AND SUMMARY

1. Quantitative determination of urinary bile pigment excretion should attempt to comprise all bile pigment compounds which could possibly be present. Methods using the diazo reaction determine bilirubin only. Therefore oxidation methods which include also bile pigments other than bilirubin are to be preferred.

2. A direct, rapid, quantitative, photo-electric method, using Hammarsten's oxidation reaction, is described. By means of an appropriate filter and by

employing the diluted acidified but untreated urine as a standard, the otherwise necessary preliminary precipitation of the bile pigments can be eliminated.

3. A comparative study of this method with three other types of quantitative determination of urinary bile pigments is reported. The methods used in this comparison were (1) the direct diazo method of Goodson and Sheard, (2) the precipitation and oxidation method of Whipple and associates, (3) the precipitation and diazo method of Scott. Urines of renal biliary fistula dogs as well as of jaundiced patients were used in this study.

4. By means of the oxidation test and by an adequate protection against superoxidation, a reliable quantitative determination of the bile pigments excreted in the urine in twenty-four hours can be obtained, although the actually excreted amounts of bilirubin may decrease considerably in the collection period.

REFERENCES

1. Kapsinow, R., Engle, L. P., and Harvey, S. C.: Intra Abdominal Biliary Exclusion From the Intestines, Surg. Gynec. & Obst. 39: 62, 1924.
2. Lemberg, R.: Animal Pigments, Ann. Rev. Biochem. 7: 421, 1938.
3. Lemberg, R., and Wyndham, R. A.: Reduction of Biliverdin to Bilirubin in Tissues, Biochem. J. 30: 1147, 1936.
4. Thierfelder, H.: Handbuch der physiologisch- und pathologisch-chemischen Analyse, Berlin, 1924, Julius Springer.
5. Goodson, W. H., and Sheard, C.: The Photelometric Determination of Bilirubin in Urine With Diazobenzene Sulfonic Acid, J. Lab. & Clin. Med. 26: 423, 1940.
6. Scott, L. C.: New Diazo Methods for Determination of Bilirubin in Blood and Urine, Brit. J. Exper. Path. 22: 17, 1941.
7. Jendrassik, L., and Czike, A.: Bestimmung des Bilirubins im Blute, Ztschr. f. d. ges. exper. Med. 60: 554, 1928.
8. Watson, C. J., Pass, I. J., and Schwartz, S.: A Study of the Supposed Conversion of Protoporphyrin to Coproporphyrin by the Liver, J. Biol. Chem. 139: 583, 1941.
9. Hooper, C. W., and Whipple, G. H.: Bile Pigment Metabolism, Am. J. Physiol. 40: 332, 1916.
10. Rous, P., and McMaster, P. D.: The Concentrating Activity of the Gallbladder, J. Exper. Med. 34: 47, 1921.
11. a. Knutti, R. E., Hawkins, W. B., and Whipple, G. H.: Hemoglobin and Bile Pigment Overproduction in the Splenectomized Bile Fistula Dog, J. Exper. Med. 61: 127, 1935.
    b. Sribhishaj, K., Hawkins, W. B., and Whipple, G. H.: Bile Pigment and Hemoglobin Interrelation in Normal Dogs, Am. J. Physiol. 96: 449, 1931.
12. Kerpolla, W.: Ein kontrolliertes Verfahren zur quantitativen Bestimmung des Bilirubins, Acta med. Scandinav. 89: 387, 1936.
13. Hammarsten, O.: Lehrbuch der physiologischen Chemie, Munich, 1922, J. F. Bergmann.
14. Rabinowitch, I. M.: Renal Threshold of Bilirubin, J. Biol. Chem. 97: 163, 1932.
15. Sivo, R., and Forrai, E.: Ueber die Zersetzlichkeit von Bilirubinloesungen, Biochem. Ztschr. 189: 168, 1927.
16. Peterman, E. A., and Cooley, T. B.: Studies on the Quantitative Estimation of Bile Pigments, J. Lab. & Clin. Med. 19: 723, 743, 1933.
17. Andrewes, C. H.: An Unexplained Diazo Color Reaction in Uremic Sera, Lancet 206: 590, 1924.
18. Harrison, G. A., and Bromfield, R. J.: The Cause of Andrewes' Diazotest for Renal Insufficiency, Biochem. J. 22: 43, 1928.
19. Becher, E.: Die Diazo—und Urochromogenreaktion im Blutfiltrat bei Niereninsuffizienz, Deutsches Arch. f. klin. Med. 148: 10, 1925.
20. Fischer, H., and Adler, E.: Ueber Mesobilirubin und das Wesen der Gmelinschen Reaktion, Hoppe Seyler's Ztschr. f. physiol. Chem. 206: 187, 1932.
21. Naumann, H. N.: A New Test for Bilirubin in the Urine, Biochem. J. 30: 762, 1936.
22. Malloy, H. T., and Evelyn, K. A.: Oxidation Method for Bilirubin Determinations in Bile and Meconium With the Photoelectric Colorimeter, J. Biol. Chem. 122: 597, 1938.

BOOK NOTICES

Hemorrhagic Diseases*

NYGAARD'S *Hemorrhagic Diseases* is a volume on blood coagulation and its mechanism, with a special discussion on the photoelectric method of determination of blood fibrin, also of the several hemorrhagic diseases. There is a discussion of vitamin K, but not of heparin.

Synopsis of Materia Medica, Toxicology and Pharmacology†

THE appearance of a second edition two years after the first speaks well for the popularity of Davison's *Synopsis of Materia Medica, Toxicology and Pharmacology.* It is rather difficult to visualize a 700 page book being a synopsis, but when compared with larger texts on the subject it emerges as truly synoptic in character. This brings us to the realization of the tremendous increase in our knowledge of the action of drugs and in the number of new drugs, in the past several years.

The volume is primarily clinical and serves as a ready reference manual.

Pathology of Trauma‡

THE pathology of trauma is a subject about which little is known to the average practitioner and medical student. Yet trauma ranks high in the list of causes of mortality and morbidity. In his book Dr. Moritz not only describes the pathology of trauma but also explains most lucidly why specific injuries take the form which they do. He calls upon an engineer's knowledge of forces, stresses, and vectors to make this clear to the reader. The book is not written from the point of view of the traumatic surgeon but from that of the pathologist and medical examiner. Consequently, it will be most valuable to persons in those fields. However, the background of knowledge which Dr. Moritz has drawn upon for the presentation of his subject is so broad that his book has a natural appeal to all physicians and students. The greater part of the book is devoted to a consideration of mechanical injuries to the various systems of the body, and emphasis is placed on the question of the predisposition of trauma to disease. This is not only a clinical problem but a most important medico-legal one, and in each instance Dr. Moritz reviews completely the evidence for and against trauma as an etiologic factor in disease.

Intelligent treatment of wounds demands a thorough knowledge of not only the anatomy but the pathology involved. The great demand today for young doctors who are trained to practice traumatic surgery on the battlefields makes it particularly fortunate that Dr. Moritz' book has appeared at this time. It is profusely illustrated, and will be a valuable teaching adjunct for those doctors preparing themselves for military duty.

---

*Hemorrha[illegible] [illegible]o-Electric Study of Blood Coagulability. By Kaare K. Nygaard, M.D., [illegible] urgery, the Mayo Foundation; Former Assistant Surgeon, the University [illegible] of the Alexander Malthe Foundation for Research in Medicine, Surge[illegible] Illustrated. Cloth, 320 pages. The C. V. Mosby Company, St. Louis, 1941.

†Synopsis of Materia Medica, Toxicology, and Pharmacology. Fo[illegible] tioners of Medicine. By Forrest Ramon Davison, B.A., M.Sc., Ph.D., [illegible] ment, The Upjohn Co., Kalamazoo, Mich. Formerly Assistant Professo[illegible] the School of Medicine, University of Arkansas, Little Rock. Second E[illegible] tions including 4 in color, 695 pages. The C. V. Mosby Company, St. Louis, 1942.

‡Pathology of Trauma. By Alan Richards Moritz, M.D. Cloth, 386 pages, with 117 illustrations, $6.00. Lea and Febiger, Philadelphia, 1942.

A Handbook of Allergy*

BLANTON'S *Handbook of Allergy* should fulfill well the purpose for which it is primarily intended. It is a synopsis type work for use by the undergraduate student, to be supplemented by personal instruction. It should also serve as a handy orientation volume for graduates who wish to become acquainted with the general subject of allergy. The discussion of treatment, both specific and symptomatic, is too abbreviated for the volume to serve adequately as a therapeutic reference aid. It is hoped that in the next edition treatment will receive more detailed discussion. When used as an undergraduate text, this becomes less of a problem since the student gains his therapeutic experience in the ward and clinic.

Directory of Medical Specialists†

THE second edition of the *Directory of Medical Specialists Certified by American Boards* is much larger than the first. A change in the page headings makes it a much more convenient reference volume. As previously, the specialties are arranged in alphabetical order. In the first edition page headings designated only the specialties. In the second edition the specialty appears on the left-hand page with the States on the right-hand and the cities scattered through the pages. This is a valuable reference volume.

Standard Nomenclature of Disease and Standard Nomenclature of Operations‡

THE *Standard Nomenclature of Disease* is now in its third edition. Many editions have been made, based primarily on the National Conference of Medical Nomenclature held under the auspices of The American Medical Association in 1940. For the first time there is also included a standard nomenclature of operations. Both sections are well indexed. Key numbers for all diseases and operations are arranged according to the punch card system so that with proper equipment any group or groups of diseases or operations may be sorted out with little expenditure of time.

The volume, published by The American Medical Association, will be especially valuable for teaching and research institutions, clinics, libraries, and statisticians.

Electrophoresis of Proteins§

ORIGINALLY a highly complicated phase of physical chemistry, electrophoresis has now entered much broader fields as an implement of investigation and has even found uses in clinical medicine. Most of us know it only by name but many are coming to realize that a more intimate acquaintance will be needed, as electrophoretic methods come to be applied in our own fields of study.

These authors describe general principles and methods of application and follow with discussion of studies of serum and plasma proteins, antibodies, antigens, enzymes, hormones, and the surface study of cells. The authors themselves have contributed a very great deal to this knowledge. The volume is necessarily still highly technical but recommended as the best reference volume on the subject that is available.

---

*A Handbook of Allergy for Students and Practitioners. By Wyndham B. Blanton, M.A., M.D., Litt. D., Professor of Clinical Medicine and Chief of the Immunology Clinic, O.P.D., Medical College of Virginia, Richmond, Virginia. Cloth, 191 pages. Charles C Thomas, Springfield, Ill., 1942.

†Directory of Medical Specialists. Certified by American Boards. 1942. Cloth, 2495 pages, $7.00. Published for the Advisory Board for Medical Specialties by Columbia University Press, New York, 1942.

‡Standard Nomenclature of Disease and Standard Nomenclature of Operations. Edited by Edwin P. Jordan, M.D. Cloth, 1022 pages. The American Medical Association, Chicago, 1942.

§Electrophoresis of Proteins and the Chemistry of Cell Surfaces. By Harold A. Abramson, Assistant Professor of Physiology, College of Physicians and Surgeons, Columbia University; Associate in Medicine, The Mount Sinai Hospital, New York City. Laurence S. Moyer, Assistant Professor of Botany, University of Minnesota, Minneapolis, and Manuel H. Gorin, Chemical Research Supervisor, Field Research Department, Magnolia Petroleum Company, Dallas, Texas. Cloth, 341 pages, $6.00. Reinhold Publishing Corporation, New York, 1942.

Outline of Histology*

HOSKINS and Bevelander's *Outline of Histology* is a loose-leaf laboratory book printed by the new photolithograph process. There are two columns to a page, facilitating easy reading, and many splendid illustrations. There is also an abundance of blank pages for notes and drawings. The volume should find wide use in the histological laboratory.

Drug Products†

HERRICK'S volume on *Drug Products* is a four hundred odd page description and analysis of the Federal Food and Cosmetic Act, designed to assist the manufacturer, distributor, and packer of drug products. It provides adequate interpretation of this complicated statute.

Human Pathology‡

THE sixth edition of Karsner's *Pathology* scarcely needs an introduction. However, there have been many changes from previous editions, enough to require resetting of type. The author and publishers have thereby taken advantage of the opportunity to make two column pages which facilitates reading. It has been abundantly and beautifully illustrated with 460 illustrations in black and white and 24 in color.

To Our Readers

The exigencies of the war situation have made it necessary to comply with the order of the War Production Board to reduce the weight of paper previously used for the JOURNAL. While this change will not affect the printed page it will affect the quality of the halftone illustrations. We regret that we have no choice in the matter and would ask the forbearance of our readers. As soon as the restrictions are removed, we shall resume our practice of printing the JOURNAL on the previously employed heavier grade of paper.

THE C. V. MOSBY CO., Publishers.

---

*Outli[illegible] Margaret M. Hoskins, Ph.D., and Gerrit Bevelander, Ph.D., Department[illegible] of Dentistry, and The Graduate School of Arts and Science, New York [illegible]. I., 179 pages, Pt. II, 113 pages, $2.50. The C. V. Mosby Company, S[illegible]

†Drug Products, Labeling Packaging Regulation. By Arthur Donald Herrick, Member of the New York and Federal Bar. Cloth, 466 pages, $7.50. Revere Publishing Company, New York, 1942.

‡Human Pathology. By Howard T. Karsner, M.D. Professor of Pathology, Western Reserve University, Cleveland, Ohio. 460 Illustrations in black and white and 24 subjects in color on 16 plates. Sixth Edition. Cloth, 817 pages, $10.00. J. B. Lippincott Company, Philadelphia, Montreal, London, 1942.

In Memoriam

Major Robert Anthony Kilduffe, M.C.

1889-1943

In charge of Laboratories, Ashford General Hospital
White Sulphur Springs, West Virginia
Associate Editor, The Journal of
Laboratory and Clinical Medicine, since 1924
Formerly
President, The American Society of Clinical Pathologists
Editor, The American Journal of Clinical Pathology
Director of Laboratories, The Atlantic City Hospital

The Journal of Laboratory and Clinical Medicine

Vol. 28 June, 1943 No. 9

CLINICAL AND EXPERIMENTAL

THE DISTRIBUTION AND POLLINATION TIMES OF THE IMPORTANT HAY FEVER-PRODUCING PLANTS IN THE UNITED STATES*

Philip M. Gottlieb, M.D., and Erich Urbach, M.D., Philadelphia, Pa.

It is axiomatic that the precise etiologic diagnosis of hay fever requires a knowledge of the pollens to which the patient is exposed; in other words, a knowledge of the identity of those plants in the patient's vicinity which are capable of causing pollinosis, their relative prevalence, the average onset and termination of their pollinating seasons, and their pollen-producing capacity. Such information for each locality is essential in correlating the patient's symptoms with the presence of his allergens, in selecting the appropriate pollens with which to perform tests, in evaluating cases clinically diagnosed as hay fever but failing to react to tests, and in choosing the proper extracts for treatment. For practical application, information about the facts mentioned is of greater importance than the variations in the quantity of atmospheric pollen grains from day to day within the season.

Few physicians, of course, have the time, the requisite botantical training, or the facilities for carrying out pollen counts or field surveys. Hence, it is necessary to rely upon pollination studies previously completed in the same, or some comparable, locale. Although the seasons vary somewhat from year to year, depending on a number of meteorologic conditions (see below), they are usually sufficiently constant for the experience of previous years to be applied clinically.

*From the Allergy Service of the Jewish Hospital, Philadelphia.
Received for publication, Sept. 28, 1942.

There undoubtedly exists a great deal of unpublished data in this field. Much of it is in the hands of allergists located throughout the country, and a commendable effort has recently* been made by the Committee on a Pollen Survey of the Society for the Study of Asthma and Allied Conditions,[27] to tap this rich source of material. This investigation has not yet reached the point where it is useful to the practicing physician.

In addition, scattered throughout the medical literature are a large number of carefully performed investigations which, *in toto*, nearly blanket the United States. These, to be sure, differ widely in scope and technique, some including only a single species or one related group of plants and some reporting findings regarding only a portion of the hay fever season. Most pertain to one locale only, others cover extensive regions of the country, and still others are national or nearly national in extent. Many are based on atmospheric pollen counts by gravity or other methods, some are the result of botanical surveys, a few combine these two approaches, and still fewer correlate these findings with clinical observations. Deficiencies encountered in various articles

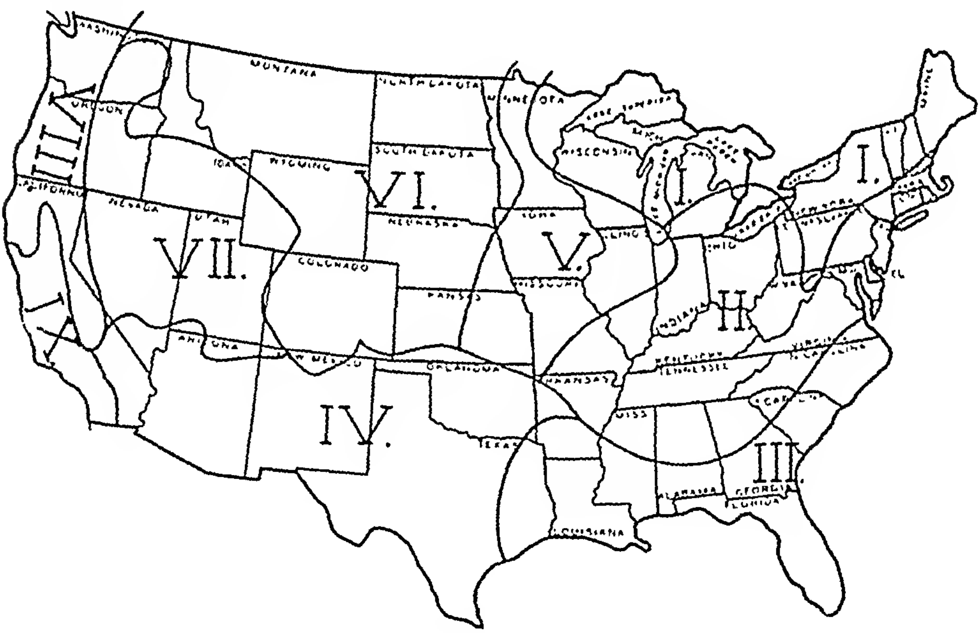


Fig. 1.—Division of the United States into nine pollination zones.

include omission of the duration of pollination and less frequently of its exact onset, grouping of several species into rough "seasons" rather than giving the separate dates, and inadequate identification of plants by ambiguous common names. Many fail to state the relative importance of each plant, or where given, to mention whether it is based on pollen counts, prevalence, abundance of pollen production, or the frequency of positive skin reactions. A common failing is the assumption, unsupported by actual determinations or any apparent scientific evidence, that the findings in a given city are characteristic of a whole state or even larger area, such as New England, the Middle Atlantic States, or the South.

*At the time this paper was undertaken and started, no such study had been begun.

Despite all these shortcomings, which, of course, by no means apply to all the reports, a great deal of useful information can be gained. And inasmuch as the data for one community can properly be considered to hold for at least a limited portion of the surrounding territory, all these observations can be compared and combined to give a representative, over-all picture of the pollen situation in the United States.

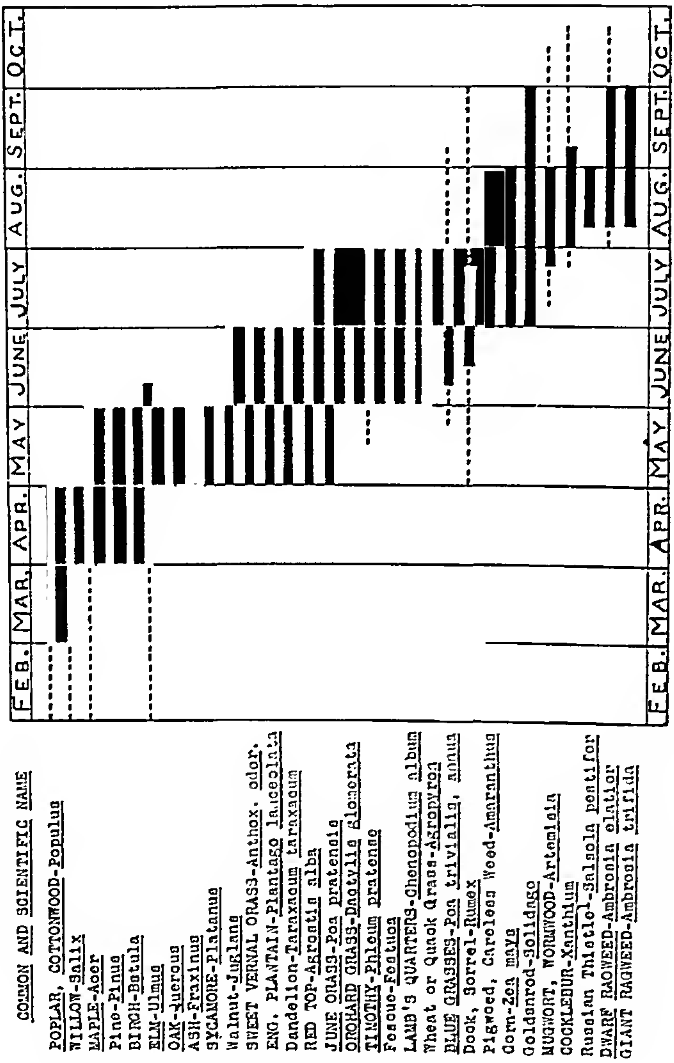


Fig. 2.—Pollination calendar for Zone I. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, Occurs in western portion of the zone only.

It is a botanical truth that probably no two species of plants precisely correspond in their distribution, range, or region of maximum growth.[1] Moreover, depending on the nature of the soil, the elevation, and a multitude of meteorologic factors, the dates of onset and cessation of pollination vary from place to place in the same area. Hopkins[2] has pointed out that, other conditions being equal, the variation in time of occurrence in temperate North America is at a general average rate of four days to each 1 degree of latitude, 5 degrees of longitude, and 400 feet of altitude, later northward, eastward, and upward

in spring and early summer, and the reverse in late summer and autumn. The average advance of the season per day is seventeen miles northward, sixty-two miles eastward, and 100 feet upward.

Nevertheless, for practical and clinical purposes, rather large districts can be considered to contain a certain type of flora with pollination seasons which, with some variation, are by and large uniform for that area. This is all the

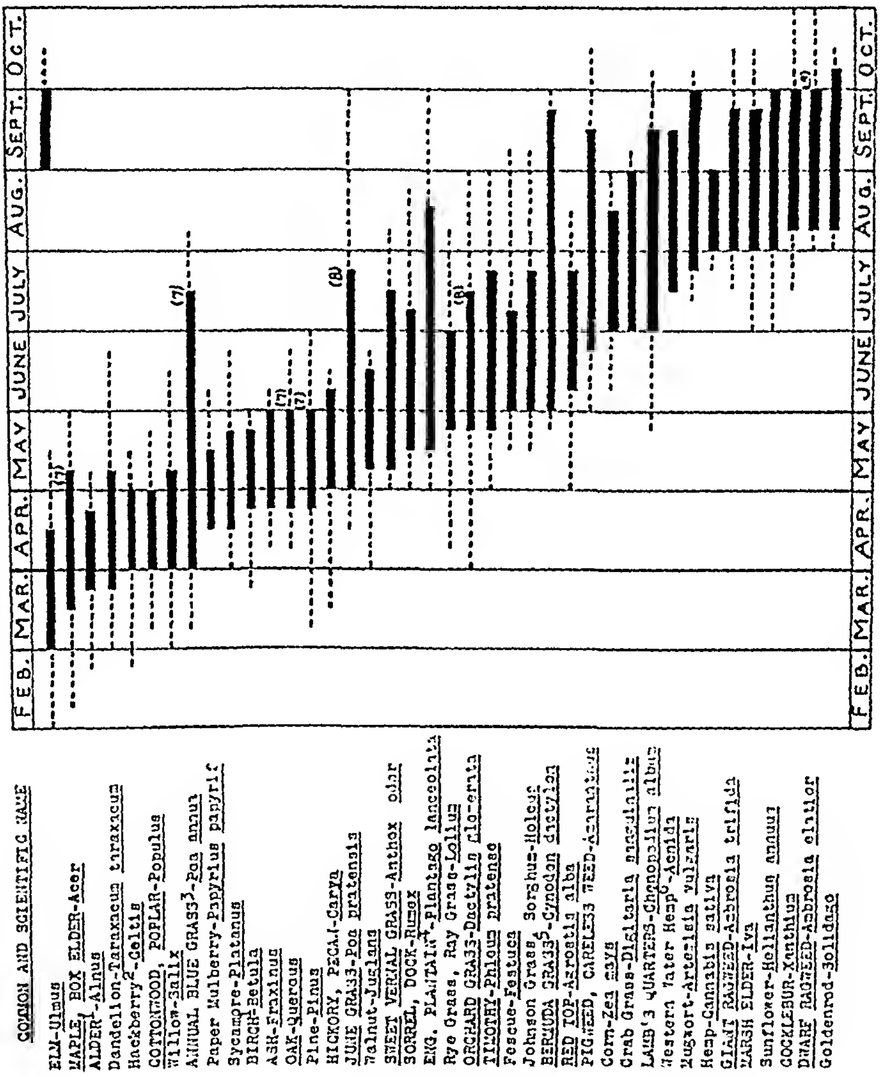


Fig. 3.—Pollination calendar for Zone II. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, Much less common in southern portion of zone; *2*, less common in northern portion; *3*, also *Poa compressa* ((Canadian blue grass) in northern portion; *4*, also *Plantago major* (common plantain), but less common; *5*, occurs in southern portion only; *6*, occurs in western portion only; *7*, pollinates one month earlier in southern portion; *8*, sometimes pollinates in January or February in southern portion; *9*, pollination commences somewhat later in southern portion.

more true in that the important hay fever-causing pollens can be carried by the wind for considerable distances, such atmospheric pollution tending to minimize the influence of local differences in plant life. It has been customary to divide the country into districts according to state boundaries or the usual geographico-economic units. A moment's thought will show that this cannot be correct for the present instance, since neither the plants themselves nor their air-borne pollens are any respectors of state borders. Even where states are bounded by rivers, the same flora is practically always established on both

banks. Hence, we have more logically resorted to the botanical-vegetational areas painstakingly delineated by Livingston and Shreve.[1] It was found necessary to modify their map to accord with more recent publications and with the

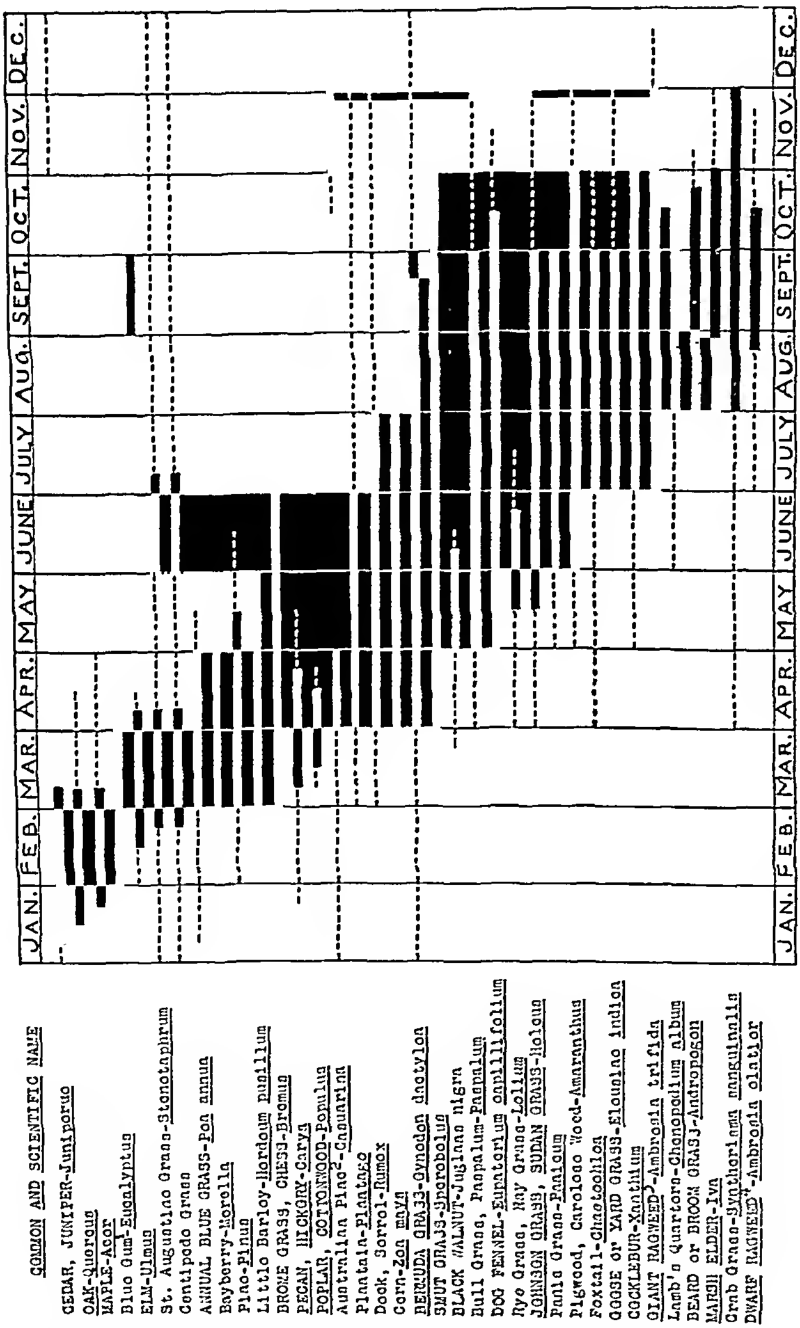


Fig. 4.—Pollination calendar for Zone III. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, Occurs in Florida only; *2*, occurs in southern Florida only; *3*, occurs chiefly in western portion—none in Florida; *4*, none in eastern Florida.

fact that we are solely concerned with plants capable of producing hay fever rather than with all the flora. Fig. 1 presents a division of the United States into nine zones according to these principles.

The literature yielded 166 articles containing usable information. Of these, forty-two covered the entire country, or at least large areas, as regards one or more species, and these are given first in the references. Parenthetically, it may

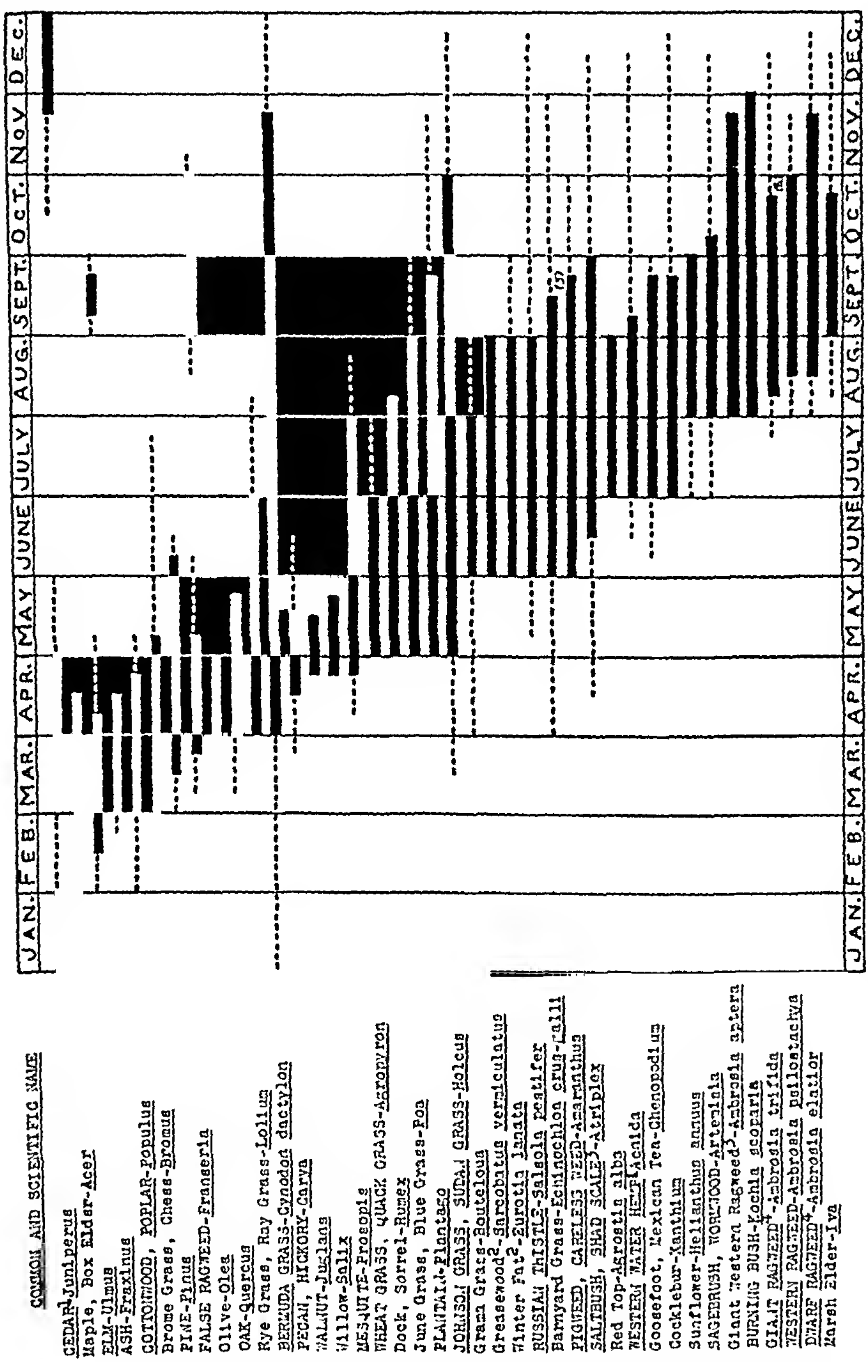


FIG. 5.—Pollination calendar for Zone IV. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. 1, Less common in western portion; 2, occurs in western portion only; 3, less common in eastern portion; 4, none in western portion; 5, pollinates perennially in protected areas; 6, pollinates earlier in extreme western Texas.

be noted that in six of them attention was confined to the West;[4, 20, 31, 36-38] in four, to the South;[8, 16, 29, 45] in four, to the Midwest;[7, 9, 22, 23] and in three, to New England and the Middle Atlantic States.[26, 39, 40] The remainder of the

articles are based on local studies or concern at most a state and are so listed. (In a few instances, portions of adjoining states were given consideration. These are entered only under the state primarily represented.) The bibliography also contains the references for Canada and Alaska.

By combining and, so to speak, "averaging" for each zone, the score or more surveys done in separate localities by various methods in different years, it is felt that a comprehensive view is obtained, which is, in some respects, possibly more accurate than the original studies themselves. The results of this method are given in Figs. 2 to 10, which constitute a pollination calendar for each of the nine zones.

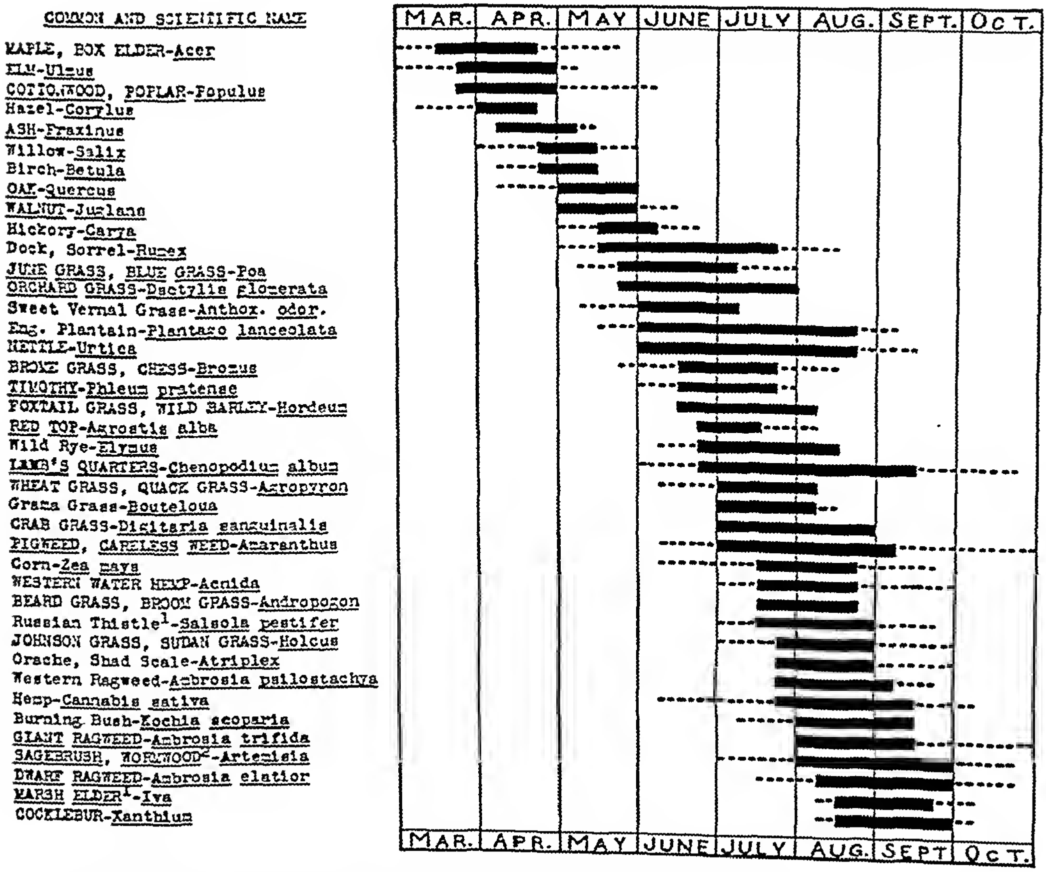


Fig. 6.—Pollination calendar for Zone V. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, None in Milwaukee, Wis.; *2*, very little in Omaha, Neb., and Milwaukee, Wis.

It must not be thought that all the plants mentioned in the literature are included in the figures. Only those of considerable importance were selected. As a matter of fact, references to nearly 300 species of weeds alone are to be found, plus hundreds of grasses and trees. The main pollination times are given in a heavy, unbroken line. This tends, in most instances, to be somewhat longer than the actual season at any one place in any one year because of the totaling of the commoner annual deviations, the combination of observations

from widely separated localities, climatic variations, differences in the time of planting of cultivated species, and other factors. This is especially true of the spring and early summer seasons rather than of late summer and fall.

It should also be pointed out that many plants pollinate sporadically both before and after the principal season, that local circumstances may influence the dates over a limited area, and that unusual weather conditions may correspondingly accelerate or delay pollination. All this is signified in the calendars by

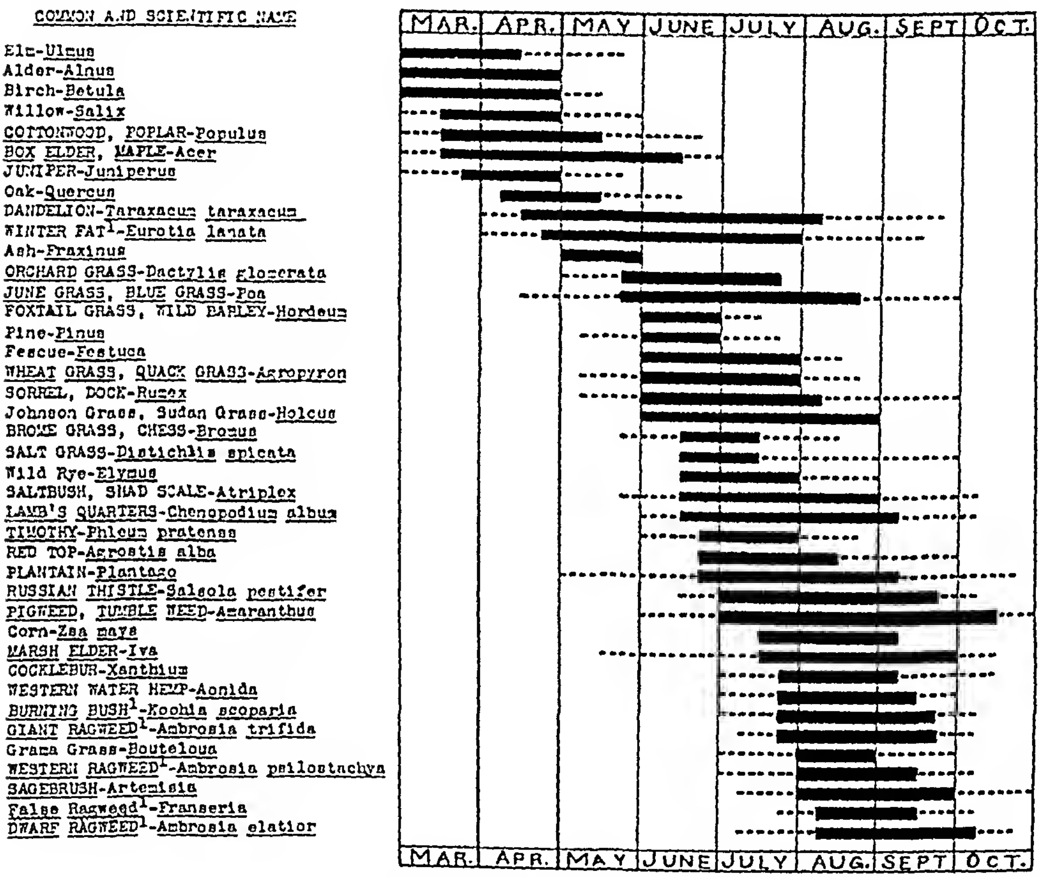


Fig. 7.—Pollination calendar for Zone VI. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, None in western Colorado, western Montana, or northern Idaho.

the finer, interrupted lines. Finally, the various trees, grasses, and weeds have been graded, according to their frequency and importance, into three groups: the major causes of hay fever, those of secondary significance, and those of lesser consequence. This, likewise, is indicated in the figures. Needless to say, other plants can, less often, be responsible for occasional cases of pollinosis, either as a result of unusual exposure or of extraordinary weather conditions. For localities near the boundary of a zone, it is suggested that the calendar for the adjacent zone also be consulted.

Naturally, certain sources of error are inherent in pollen surveys, whether by atmospheric count or botanical observation, and apply equally to this study, in so far as it is employed in any one locale.

In the first place, the beginning as well as the end of the pollination period depends, from year to year, on meteorologic factors, particularly the range of temperature, the percentage of sunshine, and the amount of precipitation. Thus, an especially warm spring and summer with heavy rainfall will appreciably advance the date of the first blooming, whereas a cold and dry preseason will delay it. The pollen calendar cannot do more than give the average dates.

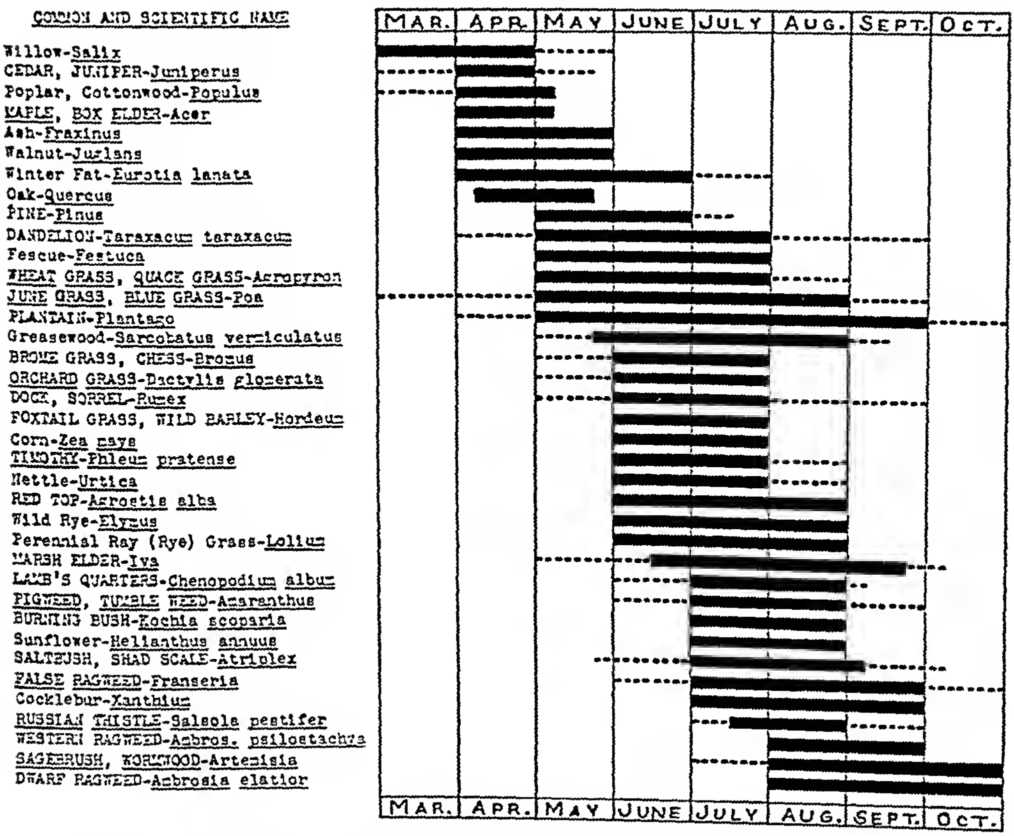


Fig. 8.—Pollination calendar for Zone VII. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively.

Fig. 11 shows how, under certain circumstances, the flowering can be hastened or retarded as much as two to four weeks. It will be seen that the onset of pollination of orchard grass (*Dactylis glomerata*) was found to vary in six consecutive years in one place from May 2 to May 29.

In the second place, extraordinary weather conditions can cause pollens which, under normal circumstances, are relatively unimportant in a given area to become major factors quite suddenly. Thus Feinberg and Durham[97] reported that in the Chicago area in 1934, unlike other years, the pollens from certain trees and from certain chenopodiales (for example, Russian thistle) were the principal allergizing agents. Duke[120] made the opposite observation that pollens which ordinarily were abundant in Kansas City, Mo., were either absent or nearly so in a season with unusual weather conditions.

Furthermore, in isolated cases, due primarily to uncommon conditions of exposure, individuals may be allergized by the pollen of trees, flowers, and

bushes which only very rarely cause hay fever. For example, Dutton[149] and Phillips[55] showed that in the fields near El Paso, Texas, and Phoenix, Ariz., respectively, where the common sugar beet (*Beta vulgaris*), a member of the family Chenopodiaceae, is intensively cultivated, some cases of hay fever are due to its pollen, which is shed from early May to mid-June. Present plans to augment greatly the acreage devoted to this plant may well lead to an increase

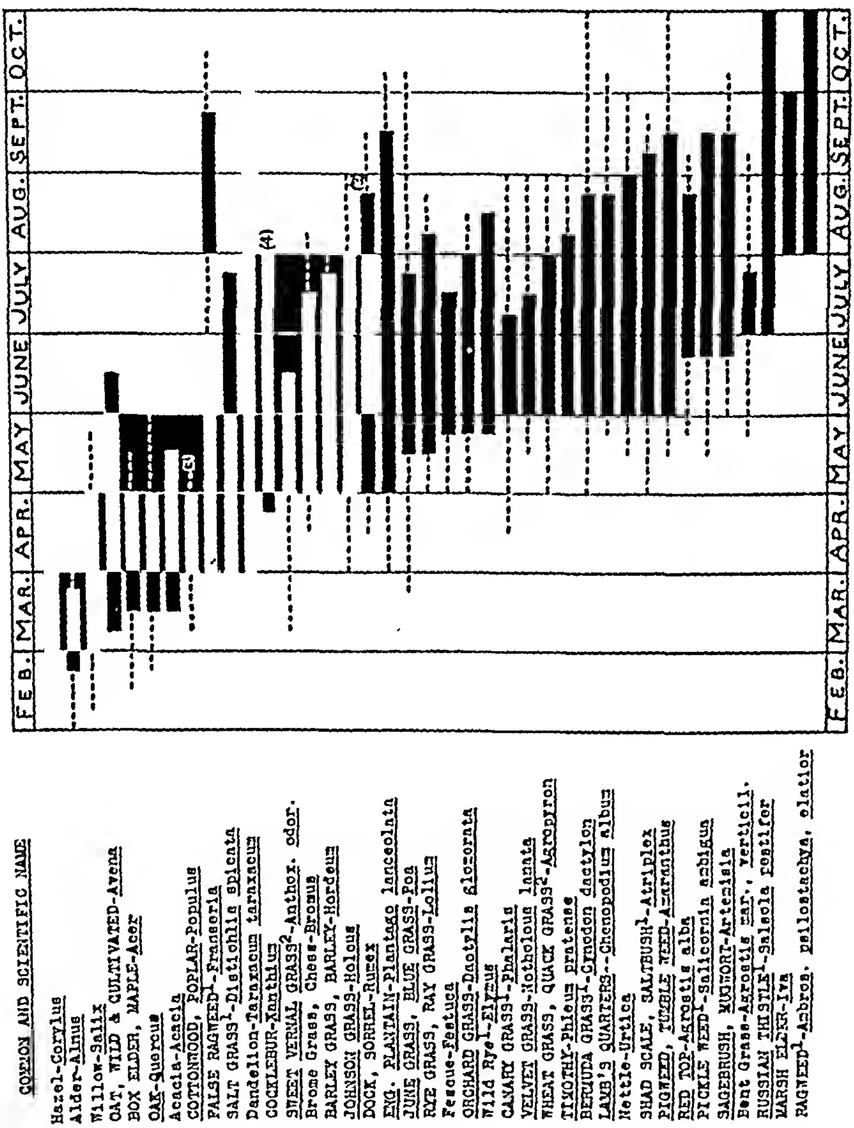


Fig. 9.—Pollination calendar for Zone VIII. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, None in northern portion; *2*, much less common in southern portion; *3*, pollinates one month earlier in southern portion; *4*, pollinates later in extreme southern portion.

in the number of such cases. McMinn and Graham[65] found that the pollen of the mirror plant (*Coprosma baueri*), an ornamental shrub widely planted in California and pollinating from the beginning of April to the end of June, yields positive skin tests in a considerable group of patients with hay fever. Langley[140] reported an unusual case of hypersensitiveness in a nurseryman to the pollen of an exotic plant, *Piqueria trinervia*, due to hothouse exposure through the winter. One of us (E. U.) encountered two exceptional cases in which hay fever

was evoked by the pollens of tree of heaven (*Ailanthus glandulosa*) and horse chestnut (*Aesculus hippocastanum*), respectively. Mention should also be made here of the possibility of allergy to insect-borne pollens under appropriate conditions.

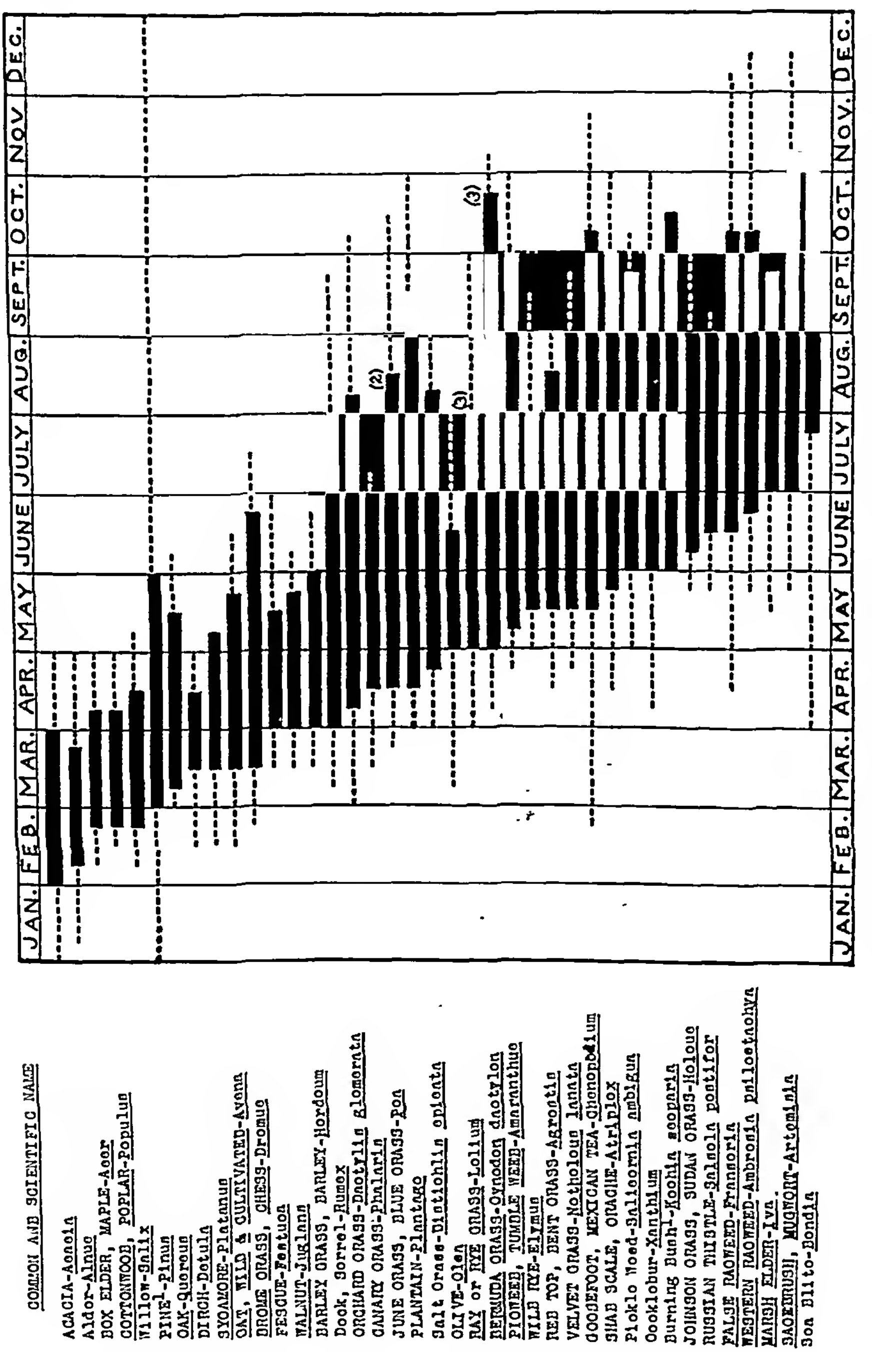


FIG. 10.—Pollination calendar for Zone IX. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type respectively. *1*, Less common in southern portion; *2*, pollinates perennially in certain areas; *3*, pollinates perennially in southern portion.

A third important source of error lies in the fact that, even within a relatively small area, differences in the elevation above sea level play a significant part in advancing or delaying the date of first pollination by a month or more.

This applies, for example, to the variations in pollination time in a valley and the adjacent mountains. As mentioned above, for each 400 feet of altitude, the delay amounts to about four days. It has also been observed (Mullin[77]) that the nature of the flora present varies greatly with increasing altitude.

Despite all these obvious and inevitable faults, such tabulations of data as the pollen calendars are certainly of great general value to the physician as a guide in the diagnosis and treatment of hay fever.

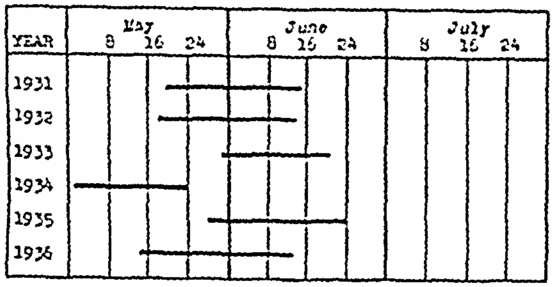


Fig. 11.—Pollination times of a single plant (orchard grass—*Dactylis glomerata*) in six successive years. Note the marked variation (as much as four weeks) in the onset and cessation of pollination in certain years.

It must be admitted that some of the plant prevalences and pollination dates presented here are not always in accord with the accepted ideas. It can only be said that they represent a careful, impartial evaluation of the published material after the elimination of the apparently less reliable observations. This accumulated information cannot be considered as final but could well be supplemented by further surveys, preferable on a nationwide scale and according to a uniform technique. The desirability of this and the locations in which it is most needed will be discussed in a separate article.[3]

SUMMARY

The necessity for a thorough knowledge of the distribution, prevalence, and pollination dates of the trees, grasses, and weeds which cause pollinosis is well known.

For this purpose, the United States has been divided into nine zones, according to botanical considerations. The plants which most commonly produce hay fever, their pollination times, and their relative importance are presented in nine pollination calendars, one for each zone.

REFERENCES

1. Livingston, B. E., and Shreve, F.: The Distribution of Vegetation in the United States as Related to Climatic Conditions, Carnegie Institution of Washington, Publication No. 284, 1921.
2. Hopkins, A. D.: Cited by Ellis, R. V., and Rosendahl, C. O.[116]
3. Gottlieb, P. M., and Urbach, E.: Pollen Surveys in the United States: A Critical Review, J. Lab. & Clin. Med. In press.

National and Regional Surveys

4. Abrams, L.: Hay Fever Plants of the Western States, Bull. Southern Pacific Gen. Hosp. 1: 162, 1932.
5. Balyeat, R. M.: Factors Which Determine the Pollen Content of the Air, J. Lab. & Clin. Med. 12: 1151, 1927.

6. Balyeat, R. M., and Rinkel, H. J.: Distribution and Importance of the Paper Mulberry, J. Allergy 3: 7, 1931.
7. Balyeat, R. M., and Stemen, T. R.: Distribution and Importance of the Acnidas As a Cause of Hay Fever and Asthma in the United States, Am. J. M. Sc. 174: 639, 1927.
8. Davison, H. M., Lowance, M. I., and Durham, O. C.: The Principal Hay Fever Plants and Pollens of the South, South. M. J. 27: 529, 1934.
9. Durham, O. C.: Typical Fall Surveys of Hay Fever Flora, J. Lab. & Clin. Med. 10: 610, 1925.
10. Idem: The Contribution of Air Analysis to the Study of Allergy, J. Lab. & Clin. Med. 13: 967, 1928.
11. Idem: Cooperative Studies in Ragweed Pollen Incidence, J. Allergy 1: 12, 1929.
12. Idem: Incidence of Ragweed Pollen in the United States During 1929, J. A. M. A. 94: 1907, 1930.
13. Idem: A Comparison of Ragweed Pollen Incidence in the United States for 1929 and 1930, J. Allergy 2: 258, 1931.
14. Idem: The Ragweed Season of 1931, J. Allergy 3: 357, 1932.
15. Idem: The Ragweed Season of 1932 in the United States, Canada and Mexico, J. Allergy 4: 105, 1933.
16. Idem: The Ragweed Situation in the Southern States, New Orleans M. & S. J. 85: 492, 1933.
17. Idem: Complicating Pollen Factors Encountered in Ragweed Hay Fever and Atmospheric Study, J. A. M. A. 100: 1846, 1933.
18. Idem: The Pollen Content of the Air in North America, J. Allergy 6: 128, 1935.
19. Idem: Pollen Studies in Selected Areas, J. A. M. A. 104: 1486, 1935.
20. Idem: Hay Fever in the Northwest, Northwestern Druggist 43: 12, 1935.
21. Idem: Evaluation of the Ragweed Hay Fever Resort Areas of North America, J. Allergy 8: 175, 1937.
22. Ellis, R. V., and Rosendahl, C. O.: Fall Hay Fever in the Upper Mississippi Valley With Special Reference to Minnesota and the Dakotas, Journal-Lancet 52: 369, 1932.
23. Feinberg, S. M.: Hay Fever in the North Central States, Illinois M. J. 61: 429, 1932.
24. Idem: Allergy in General Practice, Philadelphia, 1934, Lea & Febiger, pp. 184-235.
25. Rackemann, F. M.: Clinical Allergy, New York, 1931, The Macmillan Co., pp. 263-266.
26. Rackemann, F. M., and Smith, L. B.: Studies in Asthma. XII. The Plant Pollens of New England, J. Allergy 2: 364, 1931.
27. Report of the Committee on a Pollen Survey of the United States, The Society for the Study of Asthma and Allied Conditions, J. Allergy 13: 516, 1942.
28. Rowe, A. H.: Pine Pollen Allergy, J. Allergy 10: 377, 1939.
29. Scheppegrell, W.: Hay Fever in the Southern States, South. M. J. 9: 614, 1916.
30. Idem: The Classification of Hay-Fever Pollens from a Biological Standpoint, Boston M. & S. J. 177: 42, 1917.
31. Idem: Hay Fever: Its Cause and Prevention in the Rocky Mountain and Pacific States, Pub. Health Rep. 32: 1135, 1917.
32. Idem: Hay Fever and Its Relation to 100 of the Most Common Plants, Trees and Grasses, M. Rec. 92: 230, 1917.
33. Idem: The Seasons, Causes, and Geographical Distribution of Hay Fever and Hay Fever Resorts in the United States, Pub. Health Rep. 35: 2241, 1920.
34. Idem: Hay Fever and Asthma, Philadelphia, 1922, Lea & Febiger, pp. 171-190, 235-266.
35. Idem: The Immunological Classification of Common Hay Fever Plants and Trees, New York M. J. 117: 721, 1923.
36. Selfridge, G.: Spasmodic Vaso-Motor Disturbances of the Respiratory Tract, With Special Reference to Hay Fever, California State J. Med. 16: 164, 1918.
37. Sellers, E. D.: Pollinosis in the Southwest, South. M. J. 28: 710, 1935.
38. Stier, R. F. E., Hollister, G., and Bonser, T. A.: A Botanical Survey of Hay Fever-Producing Plants in the Pacific Northwest, J. Allergy 1: 260, 1930.
39. Thommen, A. A.: Hay Fever: The Summer Type, New York State J. Med. 30: 577, 1930.
40. Idem: Hay Fever: The Spring Type, M. J. and Rec. 131: 496, 1930.
41. Idem: In Coca, A. F. Walzer, M., and Thommen, A. A.: Asthma and Hay Fever in Theory and Practice, Springfield, Ill., 1931, Charles C Thomas, pp. 556, 694, 695.
42. Tuft, L.: Clinical Allergy, Philadelphia, 1938, W. B. Saunders Co., pp. 190-207.
43. Vaughan, W. T., and Crockett, R. W.: An Assay of Goldenrod as a Cause of Hay Fever, Ann. Int. Med. 6: 789, 1932.
44. Walker, I. C.: The Curability of Pollen Hay Fever, M. Clin. North America 19: 1547, 1936.
45. Wodehouse, R. P.: The Hay Fever Plants of the South, South. M. J. 34: 986, 1941.

Local Surveys According to States

Alabama

46. Weil, C. K.: Plants Causing Hay Fever in Alabama, J. M. A. Alabama 1: 15, 1931.
47. Idem: Hay Fever in Alabama. I. The Flora of Alabama, J. M. A. Alabama 6: 302, 1937.

Arizona

48. Lamson, R. W., and Watry, A.: Anemophilous Plants of Seligman, Arizona, J. Allergy 4: 207, 1933.
49. Idem: The Importance of the Chenopodiaceae in Pollinosis with Special Reference to Winslow and Holbrook, Arizona, J. Allergy 4: 255, 1933.
50. Idem: Survey of a Botanic Oasis in the Desert of Northern Arizona, J. Allergy 5: 389, 1934.
51. Phillips, E. W.: The Treatment of Hay Fever with Arizona Pollens, Southwestern Med. 6: 136, 1922.
52. Idem: Hay Fever in Central Arizona, Southwestern Med. 7: 273, 1923.
53. Idem: Clinical Notes on Hay Fever, Southwestern Med. 10: 4, 1926.
54. Idem: Ragweed Hay Fever in the Salt River Valley, Southwestern Med. 12: 49, 1928.
55. Idem: Ragweed Hay Fever in the Salt River Valley—Second Note, Southwestern Med. 14: 103, 1930.
56. Idem: The Changing Pollen Picture, Southwestern Med. 16: 70, 1932.
57. Idem: Pollen Incidence in Central Arizona, J. Allergy 3: 489, 1932.
58. Idem: Time Required For the Production of Hay Fever by a Newly Encountered Pollen, Sugar Beet, J. Allergy 11: 28, 1939.
59. Watson, S. H., and Kibler, C. S.: Etiology of Hay Fever in Arizona and the Southwest, J. A. M. A. 78: 719, 1922.
60. Wilson, R. A.: Some Hay Fever Problems in the Southwest, Southwestern Med. 18: 46, 1934.

California

61. Deamer, W. C., Jenkins, H. L., and Lazarus, D. S.: Pollen Survey: A Report on the Arcata District, Humboldt County, California, California & West. Med. 49: 450, 1938.
62. Deamer, W. C., and McMinn, H. E.: Studies in Atmospheric Pollen of San Francisco, J. Allergy 6: 480, 1935.
63. Hall, H. M.: Walnut Pollen as a Cause of Hay Fever, Science N. S. 47: 516, 1918.
64. Idem: Hay Fever Plants of California, Pub. Health Rep. 37: 803, 1922.
65. McMinn, H. E., and Graham, E.: Another Plant Causing Hay Fever in California: Coprosma baueri, Mirror Plant, J. Allery 8: 191, 1937.
66. Piness, G.: Study of Two Hundred and Two Cases of Hay Fever, J. A. M. A. 84: 584, 1925.
67. Piness, G., Miller, H., and McMinn, H. E.: Botanical Survey of Southern California in Relation to the Study of Allergic Diseases, Bull. South. California Acad. Sc. 25: 37, 1926.
68. Rowe, A. H.: A Study of the Atmospheric Pollen and Botanic Flora of the East Shore of San Francisco Bay, J. Lab. & Clin. Med. 13: 416, 1928.
69. Idem: Botanical Survey of San Francisco, California & West. Med. 30: 173, 1929.
70. Idem: Botanical Survey of San Joaquin County in Central California, J. Allergy 3: 375, 1932.
71. Rowe, A. H., and Howe, J. W.: A Botanical Survey of Northwestern California, J. Allergy 6: 494, 1935.
72. Stealy, C. L.: The Pollen Content of the Air of San Diego, California, J. Lab. & Clin. Med. 22: 273, 1936.
73. Watry, A., and Lamson, R. W.: Botanic Survey of Needles, California, With Special Reference to the Hay Fever-Producing Flora, J. Allergy 2: 272, 1931.

Colorado

74. Cunningham, T. D., and Wolfe, A. M.: Summary of Results of Ten Years' Hay Fever Treatment, J. Allergy 4: 48, 1932.
75. Johnson, R. P.: The Specific Treatment of Hay Fever and Pollen Asthma in Denver, Rocky Mountain M. J. 35: 305, 1938.
76. Mullin, W. V.: Pollen and Hay Fever, Tr. Am. Acad. Ophth. 27: 467, 1922.
77. Idem: Pollen and Hay-Fever, A Regional Problem, Colorado Med. 20: 96, 1923.
78. Service, W. C.: Hay Fever Flora of the Pikes Peak Region, Colorado Med. 31: 311, 1934.
79. Waring, J. J.: A Hay Fever Plant Survey of the City and County of Denver, Colorado Med. 22: 194, 1925.
80. Idem: Report of the Hay Fever Research Committee of the State Historical and Natural History Society of Colorado, Denver, Colo., 1926.
81. Wolf, J. G.: Hay Fever in Pueblo, Colorado, Colorado Med. 21: 163, 1924.

District of Columbia

82. Bernton, H. S.: Dates of Pollination of Anemophilous Plants in the District of Columbia and Vicinity, Observed in 1922, Virginia M. Monthly 50: 41, 1923.
83. Idem: Plantain Hay Fever and Asthma, J. A. M. A. 84: 944, 1925.
84. Idem: Hay Fever and Asthma Caused by the Pollen of the Paper Mulberry (Papyrius Papyrifera Kuntze), J. Lab. & Clin. Med. 13: 829, 1928.

85. Idem: Tree Pollinosis, Virginia M. Monthly 57: 170, 1930.
86. Brown, G. T.: Late Spring or Summer Hay Fever in the District of Columbia and Vicinity, Ann. Int. Med. 1: 169, 1927.
87. Idem: Seasonal Hay Fever With Special Reference to the Middle Atlantic States, J. M. Soc. New Jersey 29: 483, 1932.

Florida

88. Heinberg, C. J.: Spiny Amaranth, J. Florida M. A. 17: 221, 1930.
89. Metzger, F. C.: Hay Fever in Florida, J. Florida M. A. 19: 18, 1932.
90. Idem: The Climatic Treatment of Hay Fever and Asthma—With Special Reference to Florida, J. A. M. A. 112: 29, 1939.
91. Nichol, E. S., and Durham, O. C.: A Pollen Survey of Miami, Florida, South. M. J. 24: 947, 1931.
92. Zivitz, N.: Allergy to Australian Pine, J. Allergy 13: 314, 1942.

Idaho

93. Daubenmire, J. B.: A Qualitative and Quantitative Study of Atmospheric Pollen at Moscow, Idaho, J. Lab. & Clin. Med. 26: 311, 1940.

Illinois

94. Durham, O. C.: Hay Fever Plants and Hay Fever Pollens in Illinois, Illinois M. J. 63: 574, 1933.
95. Idem: Pollen Contamination of the Air in Illinois, Illinois M. J. 67: 475, 1935.
96. Feinberg, S. M., and Durham, O. C.: Hay Fever in Chicago and Suburbs: Clinical, Field and Air Observations, Illinois M. J. 63: 464, 1933.
97. Idem: Atypical Hay Fever Seasons: Their Significance in Treatment, Ann. Int. Med. 8: 1282, 1935.
98. Huber, H. L.: Hay Fever in the Chicago Territory, Illinois M. J. 49: 133, 1926.
99. Koessler, K. K., and Durham, O. C.: A System for an Intensive Pollen Survey With a Report of Results in Chicago, J. A. M. A. 86: 1204, 1926.
100. Taub, S. J., and Friedman, T. S.: Pollinosis, M. Clin. North America 21: 129, 1937.

Indiana

101. Durham, O. C.: Pollen Survey of Indiana, J. Indiana M. A. 22: 227, 1929.
102. Idem: Six Fall Hay Fever Seasons in Indiana, J. Indiana M. A. 28: 182, 1935.

Iowa

103. Cole, J.: A Pollen Survey in Ames, Iowa, in 1938, J. Iowa M. Soc. 29: 108, 1939.

Louisiana

104. Penfound, W. T., Efron, B. G., and Morrison, J. J.: A Survey of the Herbaceous Plants in New Orleans in Relation to Allergy, J. Allergy 1: 369, 1930.
105. Scheppegrell, W.: Analysis of Six Annual Seasons of Fall Hay Fever in New Orleans, Louisiana, Pub. Health Rep. 38: 1565, 1923.
106. Idem: Hay Fever from Tree Pollens, New Orleans M. & S. J. 78: 132, 1925.

Maine

107. Sylvester, C. B., and Durham, O. C.: Ragweed Pollen Survey in Maine for 1937, New England J. Med. 219: 428, 1938.

Maryland

108. Acquarone, P., and Gay, L. N.: A Survey of the Pollen Flora in Baltimore During 1929, J. Allergy 2: 336, 1931.
109. Patterson, P. M., and Gay, L. N.: The Pollen Content of the Air and Its Relation to Hay Fever in Baltimore, Maryland, During 1930, J. Allergy 3: 282, 1932.
110. Bubert, H. M., and Rosenberg, S.: Pollen Counts, Bull. School Med., Univ. Maryland 23: 179, 1939; 24: 239, 1940; 25: 234, 1941.

Massachusetts

111. Pratt, H. N., Colmes, A., Fromer, J., Greene, J. E., Chafee, F. H., and Clapp, W. B.: Pollen and Mold Survey of Southeastern New England—1940, New England J. Med. 225: 533, 1941.
112. Rackemann, F. M., and Smith, L. B.: The Hay Fever Plants of Eastern Massachusetts: A Preliminary Study, New England J. Med. 204: 708, 1931.
113. Idem: Ragweed Hay Fever: Symptoms and Pollen Concentration Compared (1930), New England J. Med. 204: 711, 1931.
114. Walker, I. C.: Frequent Causes and the Treatment of Seasonal Hay Fever, Arch. Int. Med. 28: 71, 1921.

Michigan

115. Waldbott, G. L., and Steere, W. C.: Survey of the Hay Fever Situation of Detroit, J. Detroit Coll. Med. & Surg. 2: 3, 1931.

Minnesota

116. Ellis, R. V., and Rosendahl, C. O.: A Survey of the Causes of Hay Fever for the State of Minnesota, Minnesota Med. 16: 379, 1933.
117. Idem: The Diagnosis and Treatment of Hay Fever With Especial Reference to Minnesota, Minnesota Med. 17: 378, 1934.
118. Rosendahl, C. O., and Dahl, A. O.: Causes, Diagnosis and Treatment of Hay Fever, Minnesota Med. 19: 505, 1936.
119. Rosendahl, C. O., Ellis, R. V., and Dahl, A. O.: Air-Borne Pollen in the Twin Cities Area With Reference to Hay Fever, Minnesota Med. 23: 619, 1940.

Missouri

120. Duke, W. W.: The Dropping Out of Pollen Seasons (Relation of the Pollen Content of the Air to Hay Fever, Asthma, and Dermatitis of the Exposed Parts), J. Allergy 2: 471, 1931.
121. Duke, W. W., and Durham, O. C.: A Botanic Survey of Kansas City, Missouri and Neighboring Rural Districts, J. A. M. A. 82: 939, 1924.
122. Idem: Pollen Content of the Air, J. A. M. A. 90: 1529, 1928.
123. Hansel, F. K.: Hay Fever: The Value of Daily Atmospheric Counts of Pollen Grains and Mold Spores in Diagnosis and Treatment, J. Missouri M. A. 37: 241, 1940.
124. Moore, G. T., and La Garde, R. V. L.: The Identification of Pollens from So-Called "Hay Fever" Plants, Ann. Missouri Bot. Gard. 13: 355, 1926.

Montana

125. Foss, A. R.: Hay Fever in Montana, Minnesota Med. 10: 587, 1927.

Nebraska

126. Maloney, E. S., and Brodkey, M. H.: Hemp Pollen Sensitivity in Omaha, Nebraska M. J. 25: 190, 1940.

Nevada

127. Albert, H., and DeBell, V.: Hay Fever in Nevada, California & West. Med. 27: 65, 1927.

New Mexico

128. Watry, A., and Lamson, R. W.: The Probable Etiology of Pollinosis in Gallup, New Mexico, J. Allergy 5: 166, 1934.

New York

129. Freund, E. M.: Perennial or Year-Round Treatment of Hay Fever With Special Reference to Pollens in the Capital District of New York State, M. Rec. 141: 357, 1935.
130. Parlato, S. J.: Studies of the Relative Intensity of Distribution of the Ragweed Pollen During a Period of Three Years, New York State J. Med. 28: 1095, 1928.
131. Spivacke, C. A.: The Treatment of Hay Fever, M. J. & Rec. 122: 223, 1925.
132. Vander Veer, A., Jr., Cooke, R. A., and Spain, W. C.: Diagnosis and Treatment of Seasonal Hay Fever, Am. J. M. Sc. 174: 101, 1927.
133. Wodehouse, R. P.: Atmospheric Pollen, J. Allergy 4: 220, 1933.

North Carolina

134. Todd, L. C.: Hay Fever Pollen Content of Air in Charlotte in 1933, South. Med. & Surg. 96: 343, 1934.

Ohio

135. Figley, K. D.: Hay Fever: Its Diagnosis and Treatment, Ohio State M. J. 22: 686, 1926.

Oklahoma

136. Balyeat, R. M.: A Survey of Oklahoma Flora, J. Oklahoma M. A. 19: 59, 1926.
137. Denny, E. R., and Tenny, G. E.: A Botanical and Pollen Survey of Tulsa County and Surrounding Territory, J. Oklahoma M. A. 25: 293, 1932.

Oregon

138. Chamberlain, C. T.: Observations on the Treatment of Hay Fever in the Pacific Northwest, Ann. Otol., Rhin. & Laryng. 36: 1083, 1927.
139. Foskett, H. H.: Some Observations on Hay Fever in the Willamette Valley, M. Sentinel 37: 565, 1929.

Pennsylvania

140. Langley, W. D.: Sensitivity to Piqueria Pollen With Case Report, J. Allergy 9: 60, 1937.
141. Scott, E. B., Criep, L. H., and Green, M. A.: Pollen Survey in Pittsburgh, Pennsylvania, 1934, J. Allergy 7: 80, 1935.

142. Tuft, L., and Blumstein, G.: Incidence and Importance of Tree Pollen Hay Fever With Particular Reference to Philadelphia and Vicinity, J. Allergy 8: 464, 1937.

Rhode Island

143. Fishbein, J. N.: Hay Fever in New England, Rhode Island M. J. 12: 36, 1929.

South Carolina

144. Hoch, J. H., and Waring, J. I.: "Hay Fever" Plants of Charleston, South Carolina and Vicinity, J. South Carolina M. A. 29: 69, 1933.

Tennessee

145. Bowie, E.: Atmospheric Pollen of Nashville, Tennessee, J. LAB. & CLIN. MED. 24: 342, 1939.
146. Henry, J. P., and Herring, A. L.: A Botanical Survey of Memphis, Tennessee and Surrounding Territory, J. Allergy 1: 163, 1930.

Texas

147. Black, J. H.: Cedar Hay Fever, J. Allergy 1: 71, 1929.
148. Black, J. H., and Durham, O. C.: Elm Pollen as a Complicating Factor in Hay Fever, J. Allergy 1: 501, 1930.
149. Dutton, L. O.: Beet Pollen and Beet Seed Dust Causing Hay Fever and Asthma, J. Allergy 9: 607, 1938.
150. French, S. W.: Pollens Which Cause Hay Fever in South Central Texas, J. Allergy 1: 286, 1930.
151. Hulsey, S.: Pollen Counts in Fort Worth, Texas for the Years 1929, 1930, and 1931, Texas State J. Med. 28: 779, 1933.
152. Kahn, I. S.: The Botany of Southwest Texas With Reference to Hay Fever and Asthma, J. A. M. A. 82: 871, 1924.
153. Idem: The Asthma Problem as it Affects the South, South. M. J. 19: 183, 1926.
154. Kahn, I. S., and Grothaus, E. M.: Studies in Pollen Sensitiveness, M. J. & Rec. 121: 664, 1925.
155. Idem: Tree Pollen Hay Fever and Asthma in the South, South. M. J. 23: 658, 1930.
156. Idem: Hay Fever and Asthma Due to Red Cedar (Juniperus virginiana) and to Mountain Cedar (Juniperus sabinoides), South. M. J. 24: 729, 1931.
157. Key, S. M.: The Etiology of Winter Hay Fever in Texas, Texas State J. Med. 13: 308, 1918.
158. Sellers, E. D.: Mesquite Tree Pollen as a Cause of Hay Fever, Texas State J. Med. 25: 297, 1929.
159. Idem: Hay Fever Pollens of the West Texas Area, Texas State J. Med. 30: 514, 1934.

Utah

160. Anderson, J. M.: Hay Fever Plants of Utah, California & West. Med. 33: 723, 1930.
161. Barrett, C. E.: Studies in Hay Fever: Clinical Observations Including a Botanical and Air Survey of the Utah Region, J. Allergy 5: 406, 1934.
162. Templeton, H. J.: Hay Fever in the Sonoran Belt of the Intermountain District, California & West. Med. 22: 317, 1924.

Virginia

163. Blanton, W. B.: Hay Fever With Comments on the Past Season in Richmond, Virginia M. Monthly 59: 140, 1932.
164. Derbes, V. J.: The Linden (Tilia) as a Factor in Seasonal Pollinosis, J. Allergy 12: 502, 1941.
165. Vaughan, W. T.: Allergy and Applied Immunology, St. Louis, 1931, The C. V. Mosby Company, pp. 70-145.
166. Vaughan, W. T.: Practice of Allergy, St. Louis, 1939, The C. V. Mosby Company, pp. 455-457, 463, 466, 468, 470.
167. Vaughan, W. T., Graham, W. R., and Crockett, R. W.: Hay Fever Pollen Prevalencies in Virginia: Review of a Six Year Survey, Virginia M. Monthly 60: 158, 1933.

Washington

168. Schonwald, P.: Atmospheric Causes of Allergy in Western Washington, Northwestern Med. 36: 14, 1937.
169. Stroh, J. E.: Flora and Pollen Surveys of Seattle and Vicinity, Northwestern Med. 39: 258, 1940.

Surveys in Canada and Alaska

170. Detweiler, H. K., and Hurst, H.: Studies on the Pollen Content of the Air, J. Allergy 1: 334, 1930.
171. Durham, O. C.: Fall Hay Fever Pollens of Canada, Canad. M. A. J. 28: 604, 1933; see also Durham.[15, 18]

172. Idem: Atmospheric Allergens in Alaska, J. Allergy 12: 307, 1941.
173. Groh, H., and Minshall, W. H.: The Ragweed Situation in Eastern Canada in Relation to Hay Fever, Canadian M. A. J. 43: 258, 1940.
174. Jamieson, H. C.: Hay Fever in Alberta, Canad. M. A. J. 36: 595, 1937.
175. LaRush, F.: The Pollen Content of the Air in Toronto, Canada, 1932, J. Allergy 5: 306, 1934.
176. MacDermot, H. E., and Howell, G. R.: The Atmospheric Pollen in Montreal, Canad. M. A. J. 34: 316, 1936.
177. Walton, C. H. A.: Pollen Disease in Western Canada, Canad. M. A. J. 42: 62, 1940.
178. Walton, C. H. A., and Dudley, M. G.: Pollen Survey in Manitoba, Canad. M. A. J. 42: 430, 1940.

SURFACE FILMS FORMED BY BLOOD PLASMA AND SERUM OF PATIENTS WITH CHRONIC ARTHRITIS*

C. WESLER SCULL, PH.D.,† AND RALPH PEMBERTON, M.S., M.D.‡
PHILADELPHIA, PA.

INTRODUCTION

PATIENTS with chronic arthritis present deviations from normal in respect to both the cellular and noncellular components of the blood. Anemia[1] is common, and moderate leucocytosis and relative increments in the proportion of aged neutrophiles are frequent. Few significant alterations from normal have been found with respect to the fasting levels of substances having small molecular weights and high solubility in water, such as sugar, urea, and electrolytes.[2] While none of the abnormalities of blood thus far described are pathognomonic for the rheumatic syndrome, they are significant in that they reflect evidence of systemic disturbances. Among newly available procedures which might be expected to throw additional light on the nature of the pathologic alterations in the fluid tissues of arthritis is one which depends upon the formation of surface films by the substances known to be present in the blood in altered amounts. Abnormal levels of globulin and albumin in the blood of certain clinically active atrophic arthritic patients have been demonstrated in this[3] and other laboratories.[4, 5] These deviations are associated with decreased suspension stability of the erythrocytes and with gelation of plasma upon the addition of formaldehyde.[6] Blood sera from comparable cases show agglutinins for hemolytic streptococci.[7]

Purified proteins and lipoids possess the quality of spreading on the clean surface of an aqueous substrate to form thin films. Such films have been extensively studied by Adam,[8] Langmuir,[9] Langmuir and Schaefer,[10, 10a, 10b] Harkins,[11] Gorter,[12, 12a] Blodgett,[13] and many others. A recent review by

*From the Department of Rheumatoid Diseases, Abington Memorial Hospital, Abington, Pennsylvania.

Supported in part by a fund in memory of Alexander B. Coxe and by a fund in memory of Dr. Angenette Parry.

Received for publication, October 1, 1942.

†Instructor in Chemistry, Assigned to Medicine, Graduate School of Medicine, University of Pennsylvania.

‡Professor of Medicine, Graduate School of Medicine, University of Pennsylvania.

Gorter[14] includes a comprehensive bibliography covering particularly studies on films produced by compounds of biologic interest. The surface films produced by various substances exhibit characteristic qualities with respect to extent, viscosity, compressibility, thickness, and structure. Such films may be formed by remarkably small amounts of material. Under appropriate conditions certain materials spread spontaneously to form films which are only one molecule in thickness. The small quantities required for observation and the characteristic qualities exhibited by physiologically important compounds, such as lipoids and proteins, suggest the possibility that the procedures developed in this field might be well adapted to the study of body fluids in health and disease.

The present report presents results of an exploratory survey made to ascertain the extent to which methods of surface film study are adaptable to clinical materials, especially as presented by patients with rheumatic diseases. A relatively simple technique based upon more elaborate methods used in the study of surface films formed by purified oils, fatty acids, other lipoids, and proteins has been devised; this reveals differences between blood fluids obtained from certain rheumatic subjects and from control subjects. A completely satisfactory interpretation of the significance of the differences encountered would require an extended and exhaustive analysis of each component fraction of the complete fluid. Such an analysis is beyond the scope of the present survey. The data available, however, disclose a new physical index of systemic deviations from normal in the arthritic patient. It appears probable that comparable deviations occur in other clinical conditions, and application of the technique described is not limited to rheumatic disorders.

CLINICAL MATERIALS

Patients from whom blood specimens were obtained for study were in the dispensary, ward or private services of the Abington Memorial Hospital, primarily because of rheumatic complaints. The general class of rheumatic disease, together with relevant records regarding the clinico-pathologic picture, is indicated with individual data on the surface films formed by the specimens.

APPARATUS

The apparatus and general procedure represent an adaptation of methods described by Langmuir.[10] Details of construction are presented, not only to indicate fully the conditions under which data were secured, but also to show that semiquantitative results can be secured even though commercial models of surface film balances or elaborate facilities for the construction of instruments are unavailable. A rectangular tray of brass was made by attaching tapped square rods (3/8 in.) with screws to the edges of a brass plate 30 × 8 × 1/8 in. A second rectangular enclosure was made by attaching similar square rods within the first at a distance of 1/4 in. from the sides and 1 in. from the ends. The second enclosure provides a water bearing which automatically guides a floating barrier used in the preparation of plates with built-up films. Blodgett[13] has demonstrated that layers of barium stearate can be used to afford a

measure of the thickness and homogeneity of films of unknown composition. The tray was fitted with a well 1 in. in diameter and 3 in. deep, 5 in. from one end of the tray. The inside of the tray was painted with black Duco, which serves several purposes; viz., seals the edges, makes materials floating on the surface of the water readily visible, and prevents contamination of the substrate by metallic ions from the tray. Before use, the inside of the tray and the edges were coated with paraffin in order to make them hydrophobic.

To facilitate measurements of area, one edge was marked off in ½ centimeter lengths. In order to provide an index of the forces exerted by films a chain balance was suspended six inches from one end of the tray. This balance consisted of a beam (made from bicycle wheel spokes), a knife edge (one-half of a safety razor blade), supported on a bearing made by depressing angles in a glass rod. Lateral forces exerted by the film were transmitted to the beam by way of a vertical extension from the beam loosely attached to a float of mica, 5¼ in. by ¾ in. In order to prevent movement of the films around the edges of the balance float and at the same time to permit the necessary freedom of motion, the following arrangement was devised. The float was fitted at each end with thin steel strips with equilateral notches, ⅛ in. Similarly notched strips (segments of safety razor blades) were attached to the top of the inner edge of the tray. A floating adaptor was constructed by fastening a piece of mica (⅜ in. by ⅛ in.) at right angles to a strip of thin steel ½ in. × ¼ in. The latter was notched by a square ⅛ in. by ⅛ in. to accommodate the mica. This adaptor was coated with paraffin and placed between the edge of the tray and the balance. While permitting movement of the float of the balance, the adaptor prevents passage of any material on the surface water around the edge of the balance. Pressure exerted by the films being transmitted to the beam can be counterbalanced and hence estimated by raising or lowering the free end of the chain. The relative length of the chain supported by the beam provides an index of pressures exerted on the float. The relative magnitude of the forces encountered were estimated by calibrating the length of chain in terms of the known forces exerted by films of oleic acid (29.5 dynes) and castor oil (15.0 dynes). The construction and appearance of the apparatus are shown in Figs. 1 and 2.

PROCEDURE

After filling the tray with distilled water to a level of about $\frac{1}{16}$ in. above the edge, the surface was swept clean by a sliding paraffin-coated chromium-plated brass rod from the balance toward each end of the tray. Materials floating on the surface were thus confined to the ends of the tray. Such sweeping was repeated four or five times. As a result of these measures the surface of the water was made essentially free from gross contamination. On standing, under conditions prevailing in our laboratory, a film of essentially constant size accumulated on the working area, as shown by moving a swing of the balance from a poised position when a barrier was advanced toward the float. Unless the area of this blank exceeded 10 sq. cm. it was assumed that there was no significant source of contamination in the tray. As a result of

observations on the blood plasma of about thirty arthritic subjects, it became apparent that the materials in approximately one cubic millimeter of plasma produced films which could be handled within the working area. In view of the difficulties involved in the direct discharge of this small volume of fluid from available measuring apparatus, it was necessary to dilute the sample. One c.c. of blood plasma or serum was diluted with 4 c.c. of 0.85 per cent sodium chloride solution. Five cubic millimeters were discharged from a differential pipette. While some encouraging results were secured by the direct transfer of a droplet of the diluted sample from a pipette to the surface of

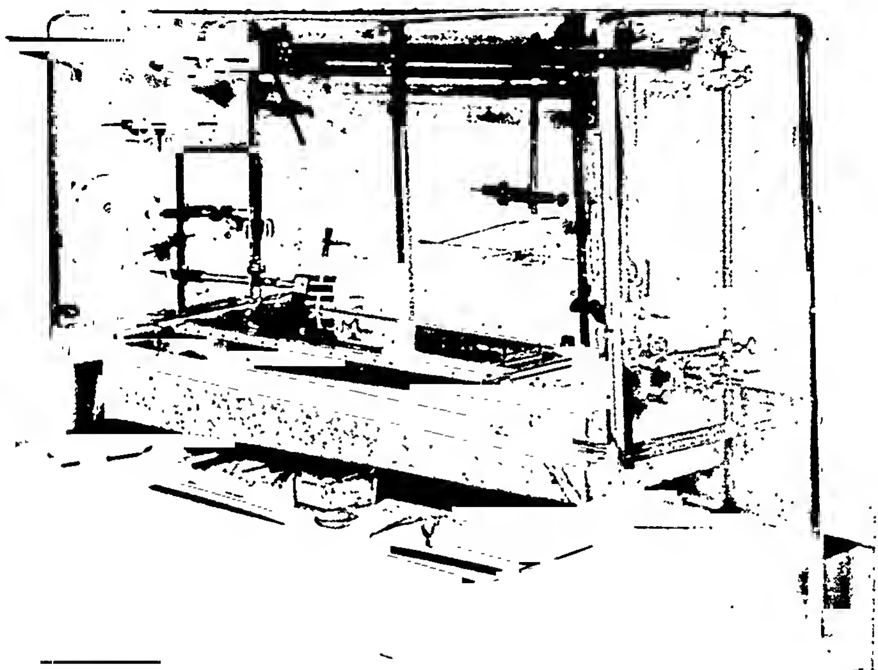


Fig. 1.—Photograph of surface film balance showing construction and arrangement of parts.

water, reproducible results were not secured with satisfactory uniformity. It was learned that more nearly reproducible data were obtained by the intermediate transfer of the diluted specimen to a metal plate. The plate held in a vertical position was then lowered cautiously through the prepared surface of water until the specimen made contact with the latter. The film-forming materials spread to the free surface of the water. After standing for five minutes the plate was dipped several times in order to insure the transfer of all of the specimen. Whether the improved reproducibility of results was actually due to this step or to increased skill in manipulation has not been established. The data presented here were secured by this procedure. The quantity of film-forming material so introduced was insufficient to cover the entire area of the tray. The films could be moved on the surface by slowly advancing the barrier toward the balance until the latter moved away from the poised po-

sition. The barrier used for maneuvering the film was fitted with a glass slide cemented on at right angles, thus functioning as a T-square. The longitudinal extent of the film was then noted, together with the weight required to counterbalance the force exerted by the film. Successive decrements of area were made by advancing the barrier by steps of ½ cm. This was followed by mak-

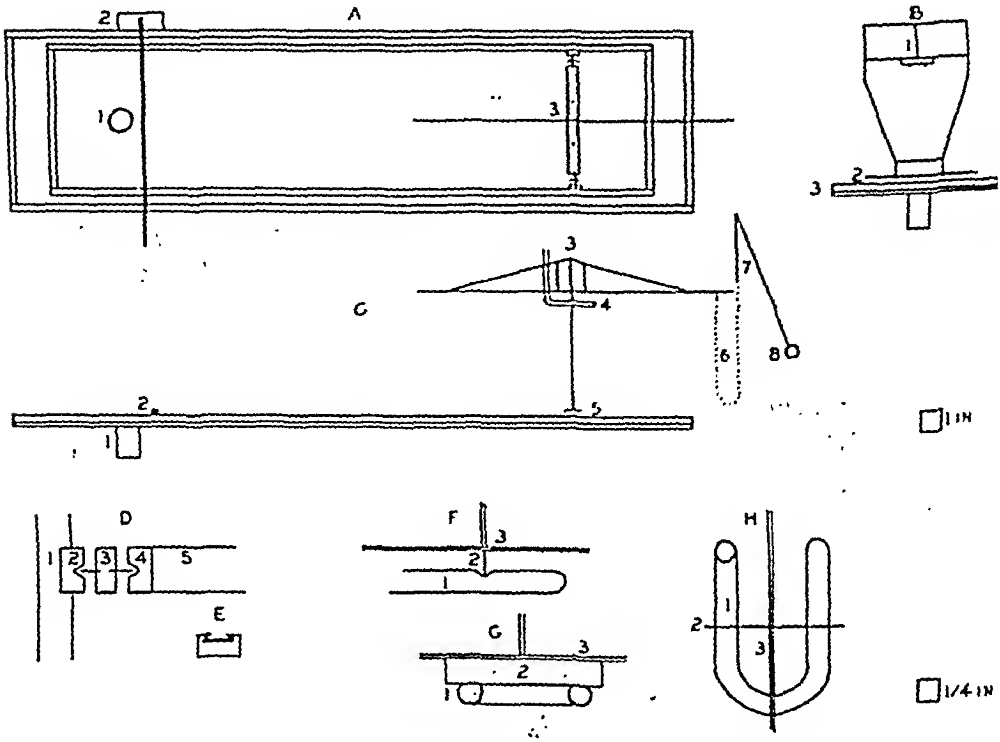


Fig. 2.—Line diagrams showing construction of surface film balance. Description:

A. Top view of balance: *1*, well; *2*, movable barrier; *3*, balance.

B. End view of balance: *1*, beam; *2*, float; *3*, tray.

C. Side view of balance: *1*, well; *2*, movable barriers; *3*, beam; *4*, support and bearing; *5*, float; *6*, chain; *7*, thread; *8*, rod windlass.

D. Top view showing attachment of float to edge of tray: *1*, edge of tray; *2*, thin steel notch; *3*, adapter; *4*, thin steel notch; *5*, mica float.

E. End view of adapter.

F. Side view of bearing: *1*, glass bearing; *2*, knife edge; *3*, beam.

G. End view of bearing: *1*, glass bearing; *2*, knife edge; *3*, beam.

H. Top view of bearing: *1*, glass bearing; *2*, knife edge; *3*, beam.

ing corresponding adjustments of the balance. Force-area charts were constructed by plotting the area in square centimeters as abscissa and the force in dynes as ordinate. The force-area diagrams so produced by different specimens were then compared by inspection.

RESULTS

In order to indicate the range of values secured by repeated determinations with the same specimens of blood, representative data are shown in Fig. 3. While the points in these instances are not identical, variations are sufficiently close to permit a satisfactory comparison of areas covered at any convenient reference pressure. Data secured with other samples were at least equally

satisfactory. It may be observed that a significant difference in area appears between the films produced by plasma and serum of the same blood. Differences of corresponding magnitude appeared between fresh and old plasmas from rapidly sedimenting bloods.

Data showing the range of representative data encountered among specimens from arthritic patients are shown in Fig. 4. While inspection reveals some differences in the slopes of the force-area curves, these are too small to be considered significant; however, the range of variations of film size are too great to be referred to variations in technique alone and must be regarded as real. Variations in area from 150 sq. cm. per 1 c.mm. plasmas to 545 sq. cm. per 1 c.mm. plasma were encountered.

Curve VC represents the force-area diagram produced by a surface film formed by plasma from a patient with the most extensive and severe active atrophic arthritis in the entire series. This patient died one month later with a pathologic diagnosis of amyloidosis with atrophic arthritis. At the time of the last observation the total protein was only 4.4 Gm. per 100 c.c. Curves CD and DL represent data from patients with active arthritis, each of whom presented sedimentation rates of 1 mm. per min., but with a total protein of 7.2 Gm. per 100 c.c. Curve PP represents another normal control subject with a normal sedimentation rate. MP represents a case of hypertrophic arthritis which is intermediate between the normal subjects and atrophic arthritic patients.

DISCUSSION

Although the initial and final portions of the force-area curves of the several films are variable, the middle portion approximates a straight line. For limited purposes it may be assumed that extrapolation of this segment of the curve to the base line represents the effective area covered by the film at "zero" pressure, or more accurately, a suitable reference pressure. Obviously, any other selected pressure within the limits of 13 to 15 dynes could be used as a reference point. It is conceivable that the first swing of the balance is produced by an extended portion of the film only or a "gaseous" film. For this reason an appreciable amount of compression is essential in order to provide assurance that the apparent area of the tray is covered by a substantial film. A constant increment of pressure with decreasing area may be regarded as indicating the formation of a reasonably stable film. The final stage, characterized by a relative decrease in pressure with diminishing area, may be attributed to collapse, folding or submersion of the film. The possibility that some components of the film are forced into solution cannot be disposed of by data at hand. The release of pressure after compression in excess of 15 dynes did not uniformly yield a film equal in area to that of the original.

It is probable that the film formed by blood fluids is composed of several substances. Certain components of blood are known to form surface films when used alone. Lipoids, i.e., fatty acids and sterols, and proteins are known to possess film-forming properties. Furthermore, surface films of mixed composition have been prepared from several of the aforementioned classes of

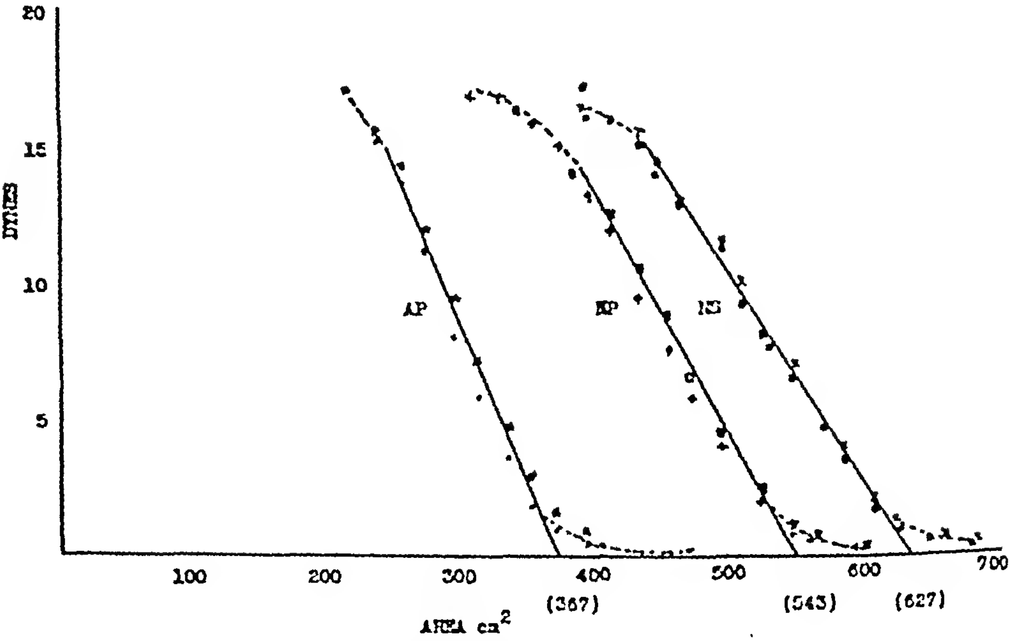


Fig. 3.—Chart showing comparative data in repeated determinations of the pressures exerted by surface films when confined to progressively decreasing areas. These films were produced from one cubic millimeter of plasma or serum from normal and arthritic subjects. AP, atrophic arthritic plasma; NP, normal plasma; NS, normal serums. From left to right, subjects: DR, WS, WS.

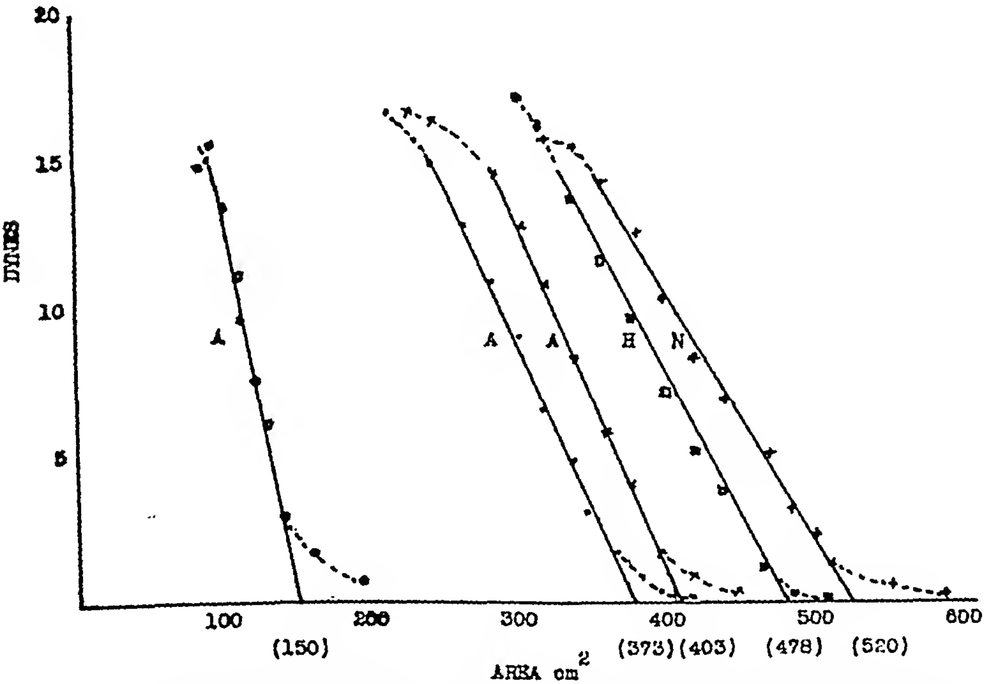


Fig. 4.—Chart showing the range of force-area curves of films produced by one cubic millimeter of blood plasma from representative arthritis: A, atrophic; H, hypertrophic; N, normal. From left to right, subjects VC, JD, DL, MP, PP.

compounds. A full account of the role of the individual compounds of the surface film formed by blood serum is beyond the scope of the present exploratory study.

The values recorded are not to be regarded as absolute and might vary with different techniques. There can be little doubt, however, as to the reality of the relative differences observed. The films formed by materials from blood serum are probably at least 25 per cent larger in area than can be accounted for by the proteins contained in them, even on the assumption that the latter are present in their most extended state. It is conceivable, however, that modifications in spreadable qualities may occur during the process of isolation of the proteins.

Another point of interest is the apparently larger areas of the films produced by serum as compared with those of plasma. This is particularly noteworthy inasmuch as fibrinogen, a spreadable material, is absent from the serum. This suggests that the reactions leading to the formation of fibrin from fibrinogen liberate materials with spreadable qualities. Determinations of film areas produced by a given plasma repeated at daily intervals revealed substantial increments. In vitro ageing of certain plasmas apparently involves a liberation of film-forming material. It is further relevant to call attention to the possible correlation of increased spreadability of serum and the suspension stability of the erythrocytes. Cells which settle rapidly in pathologic plasmas settle much more slowly in the corresponding serum and in aged plasma. These facts suggest that serum and aged plasma increase the suspension stability of red cells because of the relatively large amounts of film-forming substance as compared with the quantities present in fresh plasma. An alternative explanation based upon dilution of plasma by fluid from cells due to excessive salt concentration; viz., sodium oxalate, is not to be completely dismissed although this influence appears to be minimal.

While it may be considered possible that differences in the physical qualities of the film formed by blood serum with a high level of globulin might differ from that containing normal globulin concentrations, data presented by Gorter[11] would appear to minimize this probability. Several blood proteins, although widely different in general physical qualities, spread to form films of essentially the same magnitude. Thus both pseudoglobulin and fibrinogen form films to the extent of one square meter per milligram. Serum albumin which differs markedly from both of the former, in respect to molecular weight, electrophoretic mobility and solubility, spreads to form a film only 2 per cent less in area. Apparently such differences as may be presented by pathologic specimens are not directly related to differences in the protein components alone.

According to press reports Langmuir[15] has patented the use of the variable thickness of surface films for the diagnosis of disease. For this reason preliminary observations on the thickness of these films by the method of Blodgett,[13] although initiated, were not further pursued in the present studies.

The adaptability of the procedure to the study of clinical materials has been reasonably well established, and the method affords a practical means

of studying extremely small quantities of biologic fluids. Comparison of the spread of a mixed film with the sum of the areas of its components may afford a means of detecting otherwise unknown substances in body fluids.

A comparison of data on various arthritics with controls suggests that patients showing the greatest general systemic involvement and clinical activity present the greatest departures from normal insofar as areas of surface films are concerned. Such deviations from normal are in the direction of lowered rather than increased areas. It appears likely that certain nonarthritic patients, particularly those with lowered levels of plasma albumin, may exhibit a similar departure from normal. For this reason, deviations from normal areas of surface films are not to be regarded as satisfactory solitary indices of the arthritic process per se. Regardless of the precise interpretation to be placed upon the surface films from blood plasma of the arthritic patients, it is evident that the illness of the rheumatic patient involves more than disturbance in joints.

SUMMARY

A brief survey of an adaptation of procedures employed in the study of surface films directly to clinical materials reveals suggestive possibilities. A semiquantitative technique for the production and study of films on the surface of water from small amounts (one cubic millimeter) of blood serum and plasma has been developed. Differences have been observed with respect to the areas of films produced by equivalent volumes of normal and pathologic specimens. The areas of film formed by blood fluids from patients with rheumatic disorders are frequently smaller than those produced by blood fluids of normal subjects. One cubic millimeter of normal plasma produced films with areas of 550 sq. cm., whereas the same quantity of plasma from certain severely ill atrophic arthritic patients produced films ranging from 150 to 450 sq. cm. Films produced by plasma from less acutely ill arthritic subjects attained more nearly normal areas. These data show that the rheumatic patient suffers from a disorder of the physicochemical pattern of his blood as well as from a pathologic process in his joints.

While these deviations apparently bear a general relation to the degree of systemic involvement, they should not be regarded as pathognomonic for arthritic diseases. Some evidence is presented indicating the probability that a major factor determining the magnitude of the films relates to variations in the concentration of proteins in the specimens studied. Full interpretation of the observed differences requires more data than are now available, but there are reasonable grounds for the speculative view that certain physiologic consequences must follow upon a reduction in the "film-forming stuff" in body fluids. An evaluation of the role of film-forming capacity of blood fluids in physiologic processes and the modifications in pathologic states invites further inquiry.

REFERENCES

1. Pemberton, R.: Arthritis and Rheumatoid Conditions; Their Nature and Treatment, ed. 2, Philadelphia, 1935, Lea and Febiger.
2. Scull, C. W.: Metabolic Aspects of Rheumatoid Diseases, Pennsylvania M. J. 44: 1217, 1941.

3. Scull, C. W., Bach, T. F., and Pemberton, R.: The Serum Proteins in Arthritis. Ann. Int. Med. 12: 1463, 1939.
4. Alred-Brown, G. R. P., and Monroe, J. M. H.: The Plasma Proteins and Non-Protein Nitrogen and Sedimentation Rate in Chronic Rheumatic Disorders, Quart. J. Med. 4: 269, 1935.
5. Davis, J. S.: Protein Studies in Atrophic (Rheumatoid) and Hypertrophic Arthritis, J. Lab. & Clin. Med. 21: 478, 1936.
6. Scull, C. W., and Pemberton, R.: The Formol-Gel Reaction in Blood of Patients With Chronic Rheumatoid Diseases, Ann. Int. Med. 14: 993, 1940.
7. Dawson, M. H., and Olmstead, M.: Agglutination and Precipitin Reactions in Rheumatoid Arthritis with Hemolytic Streptococci, Groups A and G., Proc. Soc. Exper. Biol. & Med. 34: 80, 1936.
8. Adam, N. K.: Physics and Chemistry of Surfaces, ed. 2, London, 1938, Clarendon Press.
9. Langmuir, I.: Overturning and Anchoring of Monolayers, p. 9 of Recent Advances in Surface Chemistry and Chemical Physics, Lancaster, 1939, The Science Press.
10. Langmuir, I., and Schaefer, V. J.: The Effect of Dissolved Salts on Insoluble Monolayers, J. Am. Chem. Soc. 59: 2400, 1937.
10a. Langmuir, I., and Schaefer, V. J.: Properties and Structures of Protein Monolayers, Chem. Rev. 24: 181, 1939.
10b. Langmuir, I., Schaefer, V. J., and Wrinch, D. M.: Built-up Films of Proteins and Their Properties, Science 85: 76, 1937.
11. Harkins, W. D.: Some Aspects of Surface Chemistry Fundamental for Biology, p. 19 of Recent Advances in Surface Chemistry and Chemical Physics, Lancaster, 1939, The Science Press.
12. Gorter, E.: Spreading in a Monomolecular Film, Am. J. Dis. Child. 47: 945, 1934.
12a. Gorter, E.: Surface Tension and Films, Chap. 9, Chemistry Amino Acids and Proteins, C. L. A. Schmidt, ed. Springfield, 1938, Charles C Thomas.
13. Blodgett, K.: Properties of Built-up Films of Barium Stearate, J. Phys. Chem. 41: 975, 1937.
14. Gorter, E.: Properties of Protein Monolayers, Ann. Rev. Biochem. 10: 619, 1941.
15. Langmuir, I.: Colors of Thin Films Used to Detect Viruses, cited in Science News-Letter, p. 164, March 15, 1941.

VITAMIN A AND THE DETOXICATION OF MONOBROMOBENZENE*

FRANK L. HALEY, M.D., AND GEORGE S. SAMUELSEN, M.A., BROOKLYN, N. Y.

THE vital relationship of cystine to insulin, glutathione and other functionally important sulfur compounds, led us to experiment with monobromobenzene, which according to White and Jackson[1] and Stekol,[2] is specifically detoxicated by the sulfur containing amino acids, removing them from the normal nutritional processes.

In our experiments,[3] animals were fed quantities of monobromobenzene far in excess of the amount that could have been detoxicated if all of the sulfur compounds of the diet were utilized for that purpose, and led us to suspect other modes of detoxication.

Goerner[4] working in this laboratory has shown that certain carcinogenic hydrocarbons cause a rapid depletion of the liver vitamin A. This suggests that this vitamin may play a role in the detoxication of aromatic hydrocarbons and related compounds.

*From the Department of Biological Chemistry, Long Island College of Medicine, Brooklyn, N. Y.
Received for publication, October 8, 1942.

In this work we have attempted to show a relationship of the vitamin A content of liver tissue to the detoxication of monobromobenzene.

EXPERIMENTAL

The experimental work is divided into three phases. The first phase is a study of the effect of injected monobromobenzene on vitamin A deficient rats; the second, the feeding of monobromobenzene to normal rats and determining its effect upon the liver vitamin A level; and the third, the measuring of the effect of subcutaneous injection of monobromobenzene at various levels over various time intervals upon the liver vitamin A level.

Experiment No. 1.—A small series[12] of white rats that had been depleted of vitamin A by the U.S.P. method and an equal number of normal control animals were given subcutaneous injections of 100 mg. of monobromobenzene in the form of a 10 per cent solution in corn oil.

All of the rats in the vitamin A depleted group died within 48 hours after injection, while the normal animals showed no harmful effects.

Experiment No. 2.—A group of 20 adult white rats, designated A, were placed upon a normal laboratory feed (0.54 per cent S) that had been supplemented with 2 per cent of monobromobenzene. The monobromobenzene was incorporated by mixing it with twice its weight of corn oil before adding it to the stock diet. The prepared diet was stored in airtight containers. An equal number of control rats, designated B, were maintained on the unsupplemented stock diet. At intervals varying from three weeks to three months a few animals from each group were killed and their livers assayed for vitamin A content. The vitamin A was estimated by extraction according to the method of Moore[5] and the vitamin determined by the Andersen and Levine[6] modification of the method of Rosenthal and Erdelyi,[7] using a biologically standardized vitamin A concentrate as a standard for this colorimetric procedure.

The results of this experiment are tabulated in Table I.

Experiment No. 3.—Four series of adult white rats were placed upon a normal complete diet and given subcutaneous injections of monobromobenzene dissolved in corn oil (50 mg. per c.c. of oil).

Series A received 50 mg., series B 100 mg., series C 150 mg., of monobromobenzene, and series D 2 c.c. of corn oil without monobromobenzene.

The injections of monobromobenzene were made three times each week for a four-week period. At the end of this time the animals in all four series were killed and their livers assayed for vitamin A content (Table II).

DISCUSSION OF RESULTS

The first experiment shows that animals depleted of vitamin A have an extremely low capacity to withstand the toxic effect of monobromobenzene. This poor resistance cannot be entirely related to the absence of vitamin A, since such animals are in poor physical condition.

In the second experiment the lower vitamin content of the animals receiving the monobromobenzene is definite evidence of a harmful effect of this agent.

TABLE I

GROUP A			GROUP B		
RAT NO.	NUMBER OF DAYS ON DIET	TOTAL VITAMIN A CONTENT OF LIVER	RAT NO.	NUMBER OF DAYS ON DIET	TOTAL VITAMIN A CONTENT OF LIVER
1.	21	1130 units	21.	21	990 units
2.	21	650 units	22.	21	700 units
3.	28	1700 units	23.	28	1150 units
4.	28	1050 units	24.	28	1100 units
5.	35	460 units	25.	35	1160 units
6.	35	650 units	26.	35	870 units
7.	42	900 units	27.	42	730 units
8.	42	150 units	28.	42	650 units
9.	50	870 units	29.	50	1010 units
10.	50	800 units	30.	50	950 units
11.	62	55 units	31.	62	750 units
12.	62	620 units	32.	62	1170 units
13.	71	1040 units	33.	71	650 units
14.	71	610 units	34.	71	800 units
15.	78	630 units	35.	78	850 units
16.	78	220 units	36.	78	850 units
17.	85	380 units	37.	85	940 units
18.	85	140 units	38.	85	620 units
19.	92	400 units	39.	92	880 units
20.	92	350 units	40.	92	1030 units

Group A received 2 per cent monobromobenzene in diet.
Group A lost 0.11 grams per rat per day while on diet.
Group B lost 0.06 grams per rat per day on the normal diet.

TABLE II

SERIES A		SERIES B		SERIES C		SERIES D	
RAT NO.	TOTAL LIVER VITAMIN A	RAT NO.	TOTAL LIVER VITAMIN A	RAT NO.	TOTAL LIVER VITAMIN A	RAT NO.	TOTAL LIVER VITAMIN A
51.	760 units	61.	440 units	71.	800 units	81.	880 units
52.	830 units	62.	760 units	72.	1260 units	82.	750 units
53.	270 units	63.	910 units	73.	850 units	83.	910 units
54.	680 units	64.	900 units	74.	1020 units	84.	390 units
55.	1100 units	65.	650 units	75.	800 units	85.	1100 units
56.	940 units	66.	870 units	76.	590 units	86.	850 units

Series A received 50 mg. monobromobenzene three times each week.
Series B received 100 mg. monobromobenzene three times each week.
Series C received 150 mg. monobromobenzene three times each week.
Series D received no monobromobenzene.

It cannot be assumed, however, that the vitamin decrease is directly due to its action in detoxifying the monobromobenzene, since there is the possibility that the monobromobenzene interferes with the absorption of the vitamin of the diet. The fact that only minor weight changes occurred during the experimental period proves that any interference with the absorption processes is not extensive.

The injection procedure of the third experiment did not cause a noticeable change in the liver vitamin A level and proves the lower vitamin A levels obtained in the second experiment are not due to a detoxication process but are possibly associated with a failure to absorb the vitamin from the intestinal tract in the presence of the monobromobenzene.

SUMMARY

Vitamin A does not appear to be associated with the detoxication of monobromobenzene, although vitamin A deficient animals have a lowered resistance to this toxic agent.

There is some evidence that monobromobenzene interferes with the absorption of vitamin A.

REFERENCES

1. White, A., and Jackson, R. W.: Effect of Bromobenzene on Utilization of Cystine and Methionine by Growing Rats, J. Biol. Chem. 111: 507-513, 1935.
2. Stekol, J. A.: Studies on Mercapturic Acid Synthesis in Animals: Bromobenzene and *l*-Cystine in Relation to Growth of Rats on Navy Bean Meal Diet, J. Biol. Chem. 122: 55-57, 1937.
3. Haley, F. L., and Samuelsen, G. S.: Cystine Metabolism; Detoxication of Monobromobenzene, J. Biol. Chem. 119: 383-387, 1937.
4. Goerner, A.: Effect of Dibenzanthracene on Vitamin A and Total Lipid of Mitochondria, J. Biol. Chem. 122: 529-538, 1938.
5. Moore, A. R.: Is Cleavage Rate Function of Cytoplasm or of Nucleus? J. Exper. Biol. 10: 230-236, 1933.
6. Anderson, A. C., and Levine, V. E.: Reaction to Differentiate Vitamin A From Carotene by Means of Antimony Trichloride, Proc. Soc. Exper. Biol. & Med. 32: 737-741, 1935.
7. Rosenthal, J., and Erdelyi, J.: Eine neue Reaktion zum Nachweis und zur kolorimetrischen Bestimmung des Vitamins A, Biochem. Ztschr. 267: 119-123, 1933.

SYRINGADENITIS SUPPURATIVA TROPICALIS (A COMPLICATION OF LICHEN TROPICUS)*

HISTOLOGIC APPEARANCE AND ETIOLOGIC CONSIDERATIONS PARTICULARLY AS TO A POSSIBLE RELATIONSHIP OF ASCORBIC ACID AND CARBOHYDRATE METABOLISM

FREDERICK REISS, M.D., SHANGHAI, CHINA

THE observation has been made by competent observers that few persons in the tropics escape being affected by lichen tropicus (prickly heat). Those without prior history of attack are generally free or are mildly affected during their stay in hot climates, whereas those previously affected are usually subject to subsequent and increasingly severe attacks of this very annoying disease. Only a limited number of patients with prickly heat develop complications in the form of multiple furuncle-like lesions. Since only a few references have been made in the literature to this complication, it was considered important to present the subject not only from a general climatic-pathologic but also from a dermatologic point of view.

Without going into a detailed discussion, it should be noted that no acceptable explanation has been yet offered regarding the cause of lichen tropicus. Unna[1] considered it an inflammatory disease of the sebaceous glands but also was

*From the Division of Dermatology and Syphilology, National Medical College, Shanghai, China. (Chief: Frederick Reiss, M.D.)

Received for publication, October 21, 1942.

aware of an edematous swelling and obstruction of the epidermal cells due to disturbances of sweat secretion. Pollitzer[2] was partly in agreement with Unna[1] and he emphasized the obstruction of the flow of the sweat with subsequent sweat imbibition and swelling of the epidermal cells. Kayser[3] stressed the irritating effect of urea and sodium chloride of the sweat, whereas Ziemann[4] finds an explanation in high atmospheric humidity. Acton[5] speaks of an inflammation of the sweat glands caused by staphylococci and Smith[6] considers *Monilia* as the etiologic agent. Very little investigative work has been done to shed light upon the complications of the subsequent suppurative involvement of the sweat glands. Most textbooks make few references to it and many do not even mention the existence of these after-effects of prickly heat. Castellani[7] stated that "occasionally the vesicles may become purulent and crops of boils may develop." Manson-Bahr[8] agreed that prickly heat is a common though an indirect cause of boils; for the "breaches of surface following on the scratching it induces afford many opportunities for invasion of the micro-organisms of furuncular disease." Plehn[9] (in Mense's handbook) refers to the lack of cleanliness and scratching which may be blamed for extensive lesions and deep suppurations. In recent literature Acton and Smith[5, 6] make brief mention of its occurrence in India and Nigeria, respectively. G. Pernet's[10] histopathological interpretation of Radcliffe-Crockers case of acne agminata should, however, be mentioned, this being the first observation of similar nature. "The primary change appeared to occur about the sweat coils which were more or less disorganized by an inflammatory leucocytic infiltration. In parts, a hair follicle had also become involved and necrotic changes had taken place in the central parts of the infiltration. There is also a perivascular infiltration. The origin about the sweat gland agreed with the clinical evolution of the individual lesions, which commenced in the deeper parts and gradually came up to the surface with suppuration. The disease had started somewhat suddenly, about the end of August, 1901, while the patient was at the seaside for his holiday. No plasma cells were found by Pappenheim's method; few sections were stained for tubercle bacilli with negative results."

CLINICAL APPEARANCE OF LICHEN TROPICUS

Lichen tropicus (prickly heat) makes its appearance when the temperature is around or above 90°, in regions with high atmospheric moisture. The onset is characterized by a severely itching disseminated, follicular, periporitic vesicular eruption which the naked eye would recognize as a small follicular macule. The most favored regions are the covered parts of the body, especially the flexoral folds where often the eruption first appears. Only exceptionally is the face more severely involved than the rest of the body. The tiny vesicles soon become turbid, rupture, and occasionally an eczema-like plaque appears which adds to the already existing irritation. With the change to cool weather or when proper treatment is given, the eruption subsides. This is followed by a fine desquamation.

A Complication of Lichen Tropicus.—As already pointed out, only a limited number of people show severe involvement of the face and these are the persons who are frequently subject to a peculiar complication. Though itching may

be absent on the face or be considerably attenuated, there appears instead a growing sensation of tension over the face (rarely over the buttocks or the submammillary regions) which is followed by the appearance of numerous painful split-pea-sized and larger superficial and deep nodules (Fig. 1). These lesions at the onset are covered by shiny red skin which, after several days, may either develop a purulent top with subsequent suppuration of deeper portion of the lesions, or the lesions may disappear after four to eight weeks by absorption. Many weeks after the disappearance of the lesions there still remains a visible trace in the form of a distinct hyperpigmentation. Scarring occurs occasionally. The course of the disease is limited to from one to two and one-half months, during which time patients are incapacitated, not only on account of the un-

Fig. 1.—Profuse outbreak of nodular lesions with limited involvement of the upper and lower eyelids. Note the complete freedom of the center of the upper lip and of the chin.

Fig. 2.—Lesions are numerous on the bridge of the nose and forehead, but the periocular or perioral regions are hardly affected.

sightly appearance but at times also on account of pain and constitutional symptoms. Regional lymph nodes are slightly enlarged but never suppurative. This complication has been named syringadenitis suppurativa tropicalis.

Occurrence of the Complication.—Of twenty cases personally investigated, the face alone was affected in sixteen. The forehead and the bridge of the nose showed the severest involvement, whereas the periocular region, the upper lip, especially the center (philtrum) and the lower lip and chin were free (Fig. 2). In two cases a few nodules developed on the buttocks of two men and in two women a few lesions were observed under the breasts. In not a single instance were the axillae, the palms or the soles involved.

HISTOPATHOLOGY OF THE COMPLICATION

On account of the site of the lesions only a very limited number of women gave their consent for biopsy. Microscopic examination of the 20 cases were therefore made in only 8 instances. In four cases serial sections were made.

Epidermis.—In general the epidermis shows the following characteristic changes. In the majority, a distinct enlargement of the sweat and follicular orifices is observed. In an equal number of cases a subcorneal cavity is filled with detached, poorly staining cells, serofibrin and in later stages with leucocytes and organisms. The pustules are intraepidermal (producing splitting of the layers), the floor being composed of an edematous rete malpighi and the roof of lightly stained edematous prickle cells. The contents showed necrotic cells, leucocytes, and cocci. The surrounding area is distinctly edematous. These changes correspond to the position of sweat glands and in sections one can frequently trace an inflammatory focus above a sweat gland.

Corium.—The corium is the seat of major changes (Fig. 3). The papillary capillaries are dilated and surrounded by leucocytes and lymphocytes and occa-

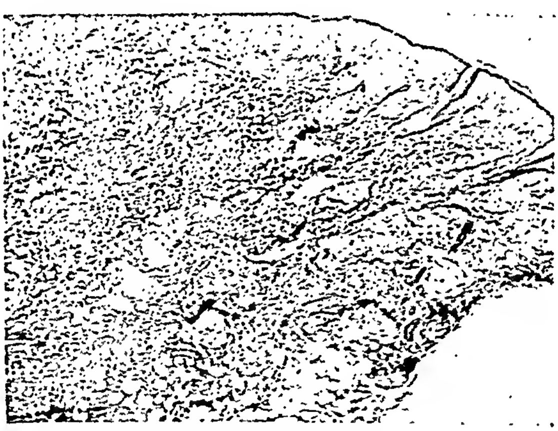


Fig. 3.—Low power photomicrograph showing: dilated pilosebaceous orifices; cellular breakdown above a sweat gland, with partial involvement of the coils.

sional plasma cells. The sweat ducts are also distinctly dilated and invaded by leucocytes and poorly staining cocci. This change increases with the beginning of the sweat duct. Not a single duct shows a cellular breakdown. The area above the sweat gland is generally the seat of cellular disintegration where small cavities are formed filled with necrotic cellular debris, leucocytes, and cocci. The coils are frequently also involved in the disintegrating process; there is no conclusive evidence as to whether the primary seat is the glandular parenchyma or whether the infection is transmitted through the duct or from the surrounding area. But it seems very suggestive that the infection travels partly via the sweat duct. Rarely, perifollicular abscesses involving the sebaceous glands are noticed. Most of the sections, however, reveal a tendency for abscess formation to develop above the sweat glands and only in rare instances is the localization a haphazard one. The cutaneous lymphatic spaces are dilated and infiltrated with inflammatory cells. At times one gains the impression as if the process would spread by the lymphatics.

CLINICAL NOTES

Our investigation of syringadenitis suppurativa tropicalis was carried out during the months of June, July, and August in 1939, 1940, and 1941 and is based on the observation of twenty Chinese patients (eighteen females, two males). The youngest was 17 years of age and the oldest 26. While this report deals only with Chinese, it should be noted that a similar complication is occasionally met with in other races in the same locality, whose occupation demands an outdoor life (at times also after long games of tennis, golf, etc.). A striking feature in our cases was the prevalence of the disease on the face and the predominating number (90 per cent) of young women (farmer, gardener class). Lack of cleanliness of the face can be safely discarded as a possible cause because the Chinese indulge in frequent wiping of their faces with towels dipped in boiling water. It could also be considered that scratching could be a contributory factor for infection. The fact, however, is the least irritated part of the skin and is hardly ever scratched. These possible exogenous etiologic factors were therefore partially eliminated and our attention was drawn to certain climatic and metabolic considerations. The staple food of the class of Chinese under our investigation consists of vegetables and polished rice. The intake of vitamin C during the summer is adequate and a nutritional insufficiency seemed unlikely. For the sake of comparison it should be mentioned that on account of the lack of green vegetables, the ascorbic acid level in the blood serum of the Chinese (in Peking) is considerably lower during winter. This, according to T. F. Yu,[11] is not due to racial difference because the serum ascorbic acid level goes up immediately as soon as ascorbic acid is added to the diet.

In tropical Java, de Haas[12] investigated the vitamin C serum level of Chinese and found that 0.86 mg. per cent was the average for 42 healthy persons. Our (unpublished) observations in healthy individuals during the summer months gave substantially the same results. We expected therefore that the excessive perspiration may cause an appreciable loss of vitamin C. This alteration may render the depleted skin susceptible to infection. Furthermore, a disturbed carbohydrate metabolism was expected, basing our supposition on the fact that an increased blood or tissue sugar content is favorable for the development of pyogenic infections of the skin. Our investigation included (1) blood serum vitamin C determination (according to Pijoan and Klemperer[13]), (2) blood sugar determinations (according to Hagedorn and Jensen).[14]

Serum Vitamin C Determinations.—Among the 20 patients investigated, the lowest level was 0.05 mg. per cent and the highest 0.78 mg. per cent. The average was 0.15 per cent. The daily administration of 200 to 300 mg. of ascorbic acid (Redoxon "Roche") increased the blood serum vitamin C level only moderately (10-20 per cent) during the period of excessive perspiration, whereas there was a definite rise with the onset of cooler weather and subsiding perspiration. This gives an indication of a possible partial pathomechanism for the vitamin C depletion and may also explain to a certain extent our difficulty in obtaining a saturation level of vitamin C during the period of excessive perspiration.

BLOOD SUGAR TOLERANCE TEST

Among the 20 eases investigated, the lowest fasting glucose level was 40 mg. per cent and the highest 70.5 mg. per eent making an average of 50.5 mg. per cent. After the intake of 1.75 Gm. of glucose per kg. of body weight, the blood sugar concentration showed a rapid rise (30-50 per eent) in the first fifteen minutes. After forty-five to sixty minutes, there was a drop of 15-20 per eent as compared with the initial rise. After two hours the level reached its highest peak with an increase of 40-50 per eent as compared with the fasting blood sugar level. After three hours, a 40-50 per eent inerease was still present. This pattern of blood sugar toleranee has been observed in 80 per eent of the cases that were studied. A typieal example is Chang Chen Chi, 17-year-old girl (O.P.D. No. 137149). (Chart 1.)

Glucose was not deteeted in a single speeimen of urine.

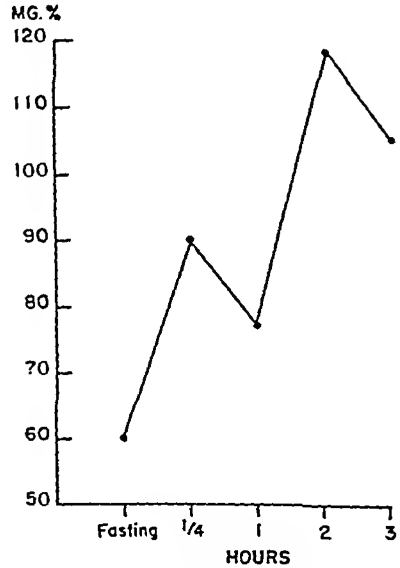


Chart 1.—Typical glucose tolerance curve.

INTERPRETATION OF DATA

In order to eorrelate the low serum ascorbic acid, with the low fasting blood sugar level, and the delayed glucose excretion, it may be of interest to recall the many investigations dealing with the relationship and with the effect of ascorbic acid on carbohydrate metabolism. It may be stated at once that there is a great variation in the reported results which is probably due to the different methods in use. While a number of investigators found that the administration of ascorbic acid to diabetics lowered the blood sugar; others such as Asinelli,[15] Mosonyi and Aszodi,[16] Stoïcesco and Gingold[17] and Tarsitano and Spena[18] found that the administration of ascorbie acid increased the level of blood sugar. Wille[19] found that vitamin C medication increased the blood sugar level especially under hypoglycaemic conditions. Stepp[20] and Schroder[21] and Altenburger[22] investigated the glycogen mobilizing effect of adrenalin. They found

a higher blood sugar peak after combined ascorbic acid medication in comparison with adrenalin alone. Kreitmair[23] confirmed these findings with his experiments on cats. Hamne[24] gave ample proof in his excellent monograph of the relationship of carbohydrates and vitamin C metabolism. According to his very carefully executed studies, the glucose tolerance curve showed an abnormal course in hypovitaminosis C. Not only was an excessive rise of blood sugar observed, but the post alimentary insulin secretion was provoked much later in comparison with cases with a normal vitamin C standard. It should be noted that animals kept on a low vitamin C diet develop a compensatory hypertrophy of the islands of Langerhans. (Rondoni and Montagnani,[25] Monasterio,[26] Borghi[27] and De Otto.[28]) These findings are parallel with the conclusions reached by Dessy and Doneddu[29] who found that both in normal persons and in diabetic patients intravenously administered ascorbic acid was recovered in greatly reduced amounts when a simultaneous intramuscular injection of insulin was given.

There is little doubt that the close interrelationship between the carbohydrate metabolism and vitamin C is firmly established. Apparently vitamin C plays in the carbohydrate metabolism the role of a bio-regulator. In other words vitamin C enters into the regulation of the blood glucose level in an important way in that it increases this level in hypoglycemia and decreases it in hyperglycemia. This interpretation would also offer a reasonable explanation for our findings.

Not only is the low fasting blood sugar level compatible and explicable with the low vitamin C blood serum level but the abnormal glucose tolerance test is a further characteristic in cases of vitamin C deficiencies. The high blood sugar curve after 2 hours indicates a lowered tolerance for carbohydrates and the persistent pathoglycemic peak after 3 hours serves as an added confirmation to the foregoing statement. The unexpected reduction of the blood sugar level after the initial rise is due to the effect of the low vitamin C level which stimulates insulin secretion (insulin effort phenomenon). This sudden effort apparently hinders the subsequent insulin secretion, which supposition is well based on the renewed rise of the blood sugar level which almost bears a certain resemblance to the blood sugar curves seen in diabetic patients. How far the adrenals are involved in this mechanism on account of the low vitamin C level, or are altered in their function on account of the enormous loss of sodium chloride through perspiration should be made a subject of further studies. It is, however, noteworthy to mention that McQuarrie[30] observed a more efficient carbohydrate utilization with a high sodium chloride medication and MacLean[31] gave evidence that the simultaneous sodium chloride medication in diabetic patients has reduced the insulin requirements.

CLIMATIC FACTORS

All of our patients lived largely an outdoor life whereby they were exposed to the effects of the sun during June, July, and August combined with the excessive atmospheric humidity and high temperature. No studies regarding climatic physiology of the skin has been carried out by us, but it is of interest to relate the experiences of Marchionini and Tor.[32] These authors have found in Anatolia (Turkey) during the summer months an enormous

increase of pyodermic infections of the face especially in individuals from ten to twenty years, females predominating. The authors offer proof that not only experimentally after ultraviolet ray exposure but also in individuals exposed to the sun, (1) the pH of the skin is shifted toward the alkaline side from 5.24 to 7.28); (2) in the dialysate of the skin the sugar, calcium and catalase increases and the amylase decreases.

Marchionini's[32] skin glucose findings, have not been correlated with blood sugar examinations. It should, however, be mentioned that low blood sugar figures do not exclude higher skin sugar findings in the same individual as the investigations of Urbach[33] have demonstrated. Whereas H. Laurens[34] states that "irradiation produces a lowered blood sugar and increases sugar tolerance." While no confirmation of Marchionini's findings has yet been reported, it seems a fair and logical working basis to explain the predisposition of the face for pyogenic infection of our cases. The shifting of the pH of the skin towards the alkaline side would certainly render the skin surface favorable for staphylococcus infection more especially in a skin in which the sugar concentration has been increased. The increase of catalase could indicate an increased oxidation-reduction which in our cases may explain the reduced vitamin C level of the blood. This depletion could furthermore be interpreted as a partial loss of excessive perspiration knowing that the sweat contains approximately 0.08-0.5 mg. per 100 c.c. and partly also in the later stages on account of the inflammatory purulent process knowing that the presence of oxidative substances in staphylococci especially demands an increased vitamin C metabolism (Harris).[35]

BACTERIOLOGIC FINDINGS

Cultures were made on 2 per cent glucose-agar from the superficial follicular vesicles, and also from the deeper lesions. In not a single case could we obtain *Monilia*, but in 19 cases *Staphylococcus aureus* was obtained in pure culture, whereas in 2 cases a scanty growth of *Staphylococcus albus* and *Staphylococcus citreus* were found.

DIFFERENTIAL DIAGNOSIS

Without previous knowledge of the clinical features, the first impression is suggestive of an iodide or bromide eruption. These can usually be ruled out because of a negative history of halogen ingestion. Against pustular syphilide are the acuteness of the lesions and the negative serologic finding. Furunculosis can be ruled out because of the absence of necrotic cores and because the localization is not strictly follicular. The symmetry is not a characteristic feature of either furunculosis or impetigo. Pyogenic infections following mosquito bites are frequent in China but favor more often the lower extremities. In all such instances, we see more or less impetiginous-crusted lesions developing at the site of scratching. From bacterids the differential diagnosis is usually not difficult because of the absence of an infectious focus and because of the unusual localization. From Harara (Heat disease of the face in Palestine) described by Dosztrovsky[36] this affection can be also differentiated because of the absence of phlebotomus in the Shanghai region, the

stinging of which is the cause of the disease. In its clinical appearance, syringadenitis suppurativa tropicalis is similar to varus nodulosus (Brook),[27] but the possibility of confusion is eliminated by the fact that varus nodulosus has a chronic course and long duration and also because of the absence of vesiculation and suppuration. Acne agminata (disseminated follicular lupus of Tilbury and Fox, acnitis of Barthelemy, hydradenitis destruens suppurativa of Politzer) should be mentioned not only because of the localization on the face, but also because of the long controversy whether the sweat-glands are the sites of primary involvement. The coppery colored lesions, their sluggish onset, the indolent nature of the disorder, the tuberculoid histologic changes are features not present in the disease under discussion in this paper.

DISCUSSION

Inflammatory and suppurative conditions affecting apocrine sweat glands are well known (Hydradenitis suppurativa). The only disease entity which concerns inflammation of the eccrine sweat glands, are the multiple abscesses of infants. Though the age incidence is entirely different, the clinical picture is similar with pea-to-hazelnut size, reddish, disseminated, hemispheric swellings which become fluctuating and discharge a greenish pus. The causative agent is *Staphylococcus albus* and the histopathology has a good many features in common with syringadenitis suppurativa tropicalis. In multiple abscesses of infants the onset is a periporitic pustule which penetrates into the deeper strata of the cutis and finally reaches the subcutis in the region of the sweat glands which at times are also involved in the process though often remain intact. In the disease under discussion in this paper it is also rather suggestive, but not quite obvious, that the process is a direct continuation of the invading infection through the sweat ducts. It is, however, also analogous that the infection may be carried from the vicinity to the sweat glands.

Pyogenic infections of the eccrine sweat glands of adults are not known in the temperate zone, and only certain climatic conditions and metabolic changes such as occurred in our patients would explain such an eventuality. The dilated sweat pores in prickly heat are not only a natural port of entrance for pyogenic cocci, but the irritation due to profuse perspiration renders the sudoriparous apparatus a locus minoris resistentiae. To this we have to add the diminished bacteriostatic power of the greatly diluted sweat and the possible changes brought about by the action of sunlight (increased sugar of the skin, etc.). While with large doses of vitamin C (900 to 1000 mg. daily) an appreciable improvement was noticed, smaller doses, 2 to 300 mg. were only effective when the hot weather subsided and perspiration decreased. The serum vitamin C and blood sugar level could only be increased with difficulty during the hot season, but both improved simultaneously with the onset of the cooler weather. These observations lead us to conclude that through the excessive loss of vitamin C the carbohydrate metabolism was disturbed. Though the fasting blood sugar level was rather low, the glucose tolerance test showed not only an abnormal high postcibal range, but also a delayed excretion of the sugar. The possibility of an increased glucose content of the skin, more

especially of the exposed parts, must be considered. The findings of Marchionini and Tor,[32] in respect to cases similar to our own, appear fairly reasonable because in our cases the facial localization was also a predominant feature and the covered parts seldom revealed lesions of a similar nature, though the frequent scratching, particularly of the covered regions affected by prickly heat, would greatly favor an infection. How far, besides the disturbed carbohydrate-vitamin C equilibrium, other colloidal chemical changes of the sweat gland parenchyma are involved is a matter of conjecture and beyond the scope of the present report.

TREATMENT

Treatment consisted of conservative measures, though in a few cases small incisions were made. Otherwise the local treatment depended chiefly on the stage of the disease. In case of pain, compresses with a soothing lotion was applied. In cases where the lesions were nearing breakdown, application of the following ointment was found effective:

Ichthyol	10.0 Gm.
Hydrarg. oxyd. rubr.	0.2 Gm.
Resorcin	0.4 Gm.
Vaseline	ad 20.0 Gm.

Internal medication included ascorbic acid, 200-1000 mg. daily. Neither the sulfonamide drugs (sulfathiazole or sulfapyridine) nor toxoid gave us any encouraging results.

CONCLUSIONS

1. An inflammatory and suppurative disorder of the eccrine sweat glands occurring in Chinese patients has been described under the name syringadenitis suppurativa tropicalis.

2. The disease is thought to be a complication of lichen tropicus (prickly heat).

3. The causative agents of the disease are assumed but not proved to be the *Staphylococci aureus* and *albus*.

4. Evidence is advanced that the inflammatory involvement and infection of the sweat glands is furthered by an excessive loss of vitamin C and altered carbohydrate metabolism.

5. Treatment consists in the use of appropriate topical applications and vitamin C internally.

I wish to express my appreciation to Dr. C. S. Pan, I. assistant of the Division of Dermatology, National Medical College, and to the Division of Physiological Sciences, Lester Institute for Medical Research for their kind assistance in the execution of the biochemical investigation.

REFERENCES

1. Unna, P. G.: Die Histopathologie der Hautkrankheiten, 916-920, 1894.
2. Pollitzer: Lichen tropicus, J. Cut. Dis. 11: 50-56, 1893.
3. Kayser, J. D.: Voordrachten over tropische Huitzikten. 1920.
4. Ziemann, H.: Lichen tropicus, Jadassohn's Handbuch der Hautkrankheiten 12: 511, 1932.
5. Acton, H. W.: Prickly Heat, Indian M. Gaz. 61: 321, 1936.
6. Smith, E. C.: Prickly Heat, Tr. Roy. Soc. Trop. Med. & Hyg. 20: 344, 1936.
7. Castellani and Chalmers: Manual of Tropical Medicine, 2224-2226, 1919.
8. Manson-Bahr, Pl. H.: Manson's Tropical Medicine, p. 634, 1936.

9. Mense, C.: Handbuch der Tropenkrankheiten, p. 6, 1905.
10. Pernet, G.: Brit. J. Dermat. 14: 131, 1902.
11. Yu, T. F.: Blood Vitamin C Content of Chinese in Peiping During the Winters of 1938 and 1939, Chinese M. J. 56: 334, 1935.
12. de Haas, J. H., and Meulemans, I. O.: Vitamin C in Blood; in Children and Adults, Geneesk. tijdschr. v. Nederl.-Indië 78: 2672, 1938.
13. Pijoan, M., and Klemperer, F.: Determination of Blood Ascorbic Acid, J. Clin. Investigation 16: 443, 1937.
14. Harrison, G. A.: Chemical Methods in Clinical Medicine, New York, 1930, The Macmillan Co., 124-127.
15. Asinelli, C.: Influenza della Vitamina C sul tasso glicemico, Gior.d.r.Accad. di med. di Torino 99: 81, 1930.
16. Mosonyi, J., and Aszodi, A.: Beeinflussung der Langerhansscher Inseln durch den Vagus mittels der Vitamine B, und C auf den Gaswechsel normaler und skorbutischer Meerschweinchen, Ztschr. f. physiol. Chem. 222: 100, 1933.
17. Stoïcesco, S., and Gingold, N.: 1. Action de l'acide ascorbique (Vitamine C) sur le métabolisme des hydrates de carbone chez l'homme normal, Bull. Acad. de méd. de Roumanie 1: 130, 1936.
2. Action de l'acide ascorbique sur le metabolism des hydrates de carbone chez les diabetiques, Bull. Acad. de méd. de Roumanie 1: 369, 1936.
3. Action de l'acide ascorbique sur le metabolisme des hydrates de carbone, Bull. Acad. de méd. de Roumanie 1: 709, 1936.
18. Tarsitano, A., and Spena, A.: Azione della Vitamina C sul tasso glicemico nei sani e negli epatopazienti, Riforma med. 52: 1271, 1936.
19. Wille, Erika: Vitamin C und Kohlehydratstoffwechsel, Deutsche med. Wchnschr. 65: 1117, 1939.
20. Stepp, W., Schroeder, H., and Altenburger, E.: Vitamin C und Blutzucker, Klin. Wchnschr. 14: 933, 1935.
21. Schroder, H.: Beziehung der wichtigsten Vitamine zum Kohlehydratstoffwechsel, Ztschr. f. d. ges. exper. Med. 101: 273, 1937.
22. Altenburger, E.: Ueber die Beziehungen der Ascorbinsäure zum Glykogenhaushalt der Leber, Klin. Wchnschr. 15: 1129, 1936.
23. Kreitmair, H.: Beiträge zur Pharmakologie der Ascorbinsäure (Vit. C), Arch. f. exper. Path. u. Pharmakol. 176: 326, 1934.
24. Hamne, B.: Studien uber die Biologie des Vit. C. mit besonderer Berucksichtigung der Kohlehydratstoffwechsels, Acta pediat. 28: suppl. IV, 1911.
25. Rondoni and Montagnani, quoted by Dessy and Doneddu. Original unobtainable.
26. Monasterio, G.: Probl. nutriz 4: 1, 1927 (quoted by Dessy and Doneddu). Original unobtainable.
27. Borghi, B.: Arch. Ist. biochem. ital. 1: 69, 1929. Quoted by Dessy and Doneddu.
28. De Otto: quoted by Dessy and Doneddu. Original unobtainable.
29. Dessy, T., and Doneddu, K.: Einfluss des Insulins auf die Vitamin C. Ausscheidung im Harn und Speichel nach intravenoser Ascorbinsaure—Belastung bei normalen und Diabetikern, Endokrinologie 23: 105, 1940.
30. McQuarrie, Irvin: The Effect of Excessive Salt Ingestion on Carbohydrate Metabolism and Arterial Pressure in Diabetic Children, Proc. Staff Meet., Mayo Clin. 10: 234, 1935.
31. MacLean, A. R.: Observation on Administration of Sodium Chloride in Diabetes, Proc. Staff Meet., Mayo Clin. 10: 321, 1933.
32. Marchionini, A., and Tor, S.: Zur Klimaphysiologie und pathologie der Haut. Aetologie der Pyodermien in Zentralanatolien, Arch. f. Dermat. u. Syph. 181: 230, 1940.
33. Urbach, E.: Bertraege zur physiolog. und path. Chemie. der Haut, Arch. f. Dermat. u. Syph. 157: 160, 1929.
34. Laurens, H.: The Physiologic Effects of Ultraviolet Radiation, J. A. M. A. 111: 26, 2385, 1938.
35. Harris, L.: Vitamin C and Infection, Perspectives of Biochemistry, London, 1939, Cambridge University Press, pp. 329.
36. Dosztrovsky, A.: Zum Wesen der "Harara," Arch. f. Schiffs- u. Tropen-Hyg. 29: 443, 1925.
37. Brook, H. G.: Varus nodulosus. Ikonographia Dermat. Fasc. 1: 15, 1906.

THE DIFFUSION OF DYES IN ETHYLENE GLYCOL GELS

FRANCES C. BROWN, PH.D., AND E. EMMET REID, PH.D.
BALTIMORE, MD.

INTRODUCTION

SCHULEMANN[13] has pointed out the relation between the diffusion of a dye and its usefulness as a tissue stain. Regardless of whether the mechanism of staining is explained by the formation of chemical compounds between the dye and the tissue[14] or by the adsorption of the dye by the tissue,[9] the rate of penetration of the dye is a factor which must be considered in the choice of a dye as a stain. Stiles[15] has shown that conditions in dilute gels duplicate closely those present in tissues. Fautrez and Lison[4] have emphasized that while diffusion in a gel is a complicated process and should not be expected to give true values of the dimensions of the diffusing particle, nevertheless diffusion in tissues and in gels are similar processes and are controlled by the same factors. Accordingly, the diffusion of dyes in gels should throw some light on the usefulness of dyes as stains.

The diffusion of certain acid and basic dyes in ethylene glycol gels has been measured. Among the acid dyes investigated were: orange II, mercurochrome, metanil yellow, congo red, and pontamine blue BBF. Chrysoidine Y was chosen as a representative of the basic dyes. Diffusion took place in 5 per cent gelatin gels of 80 per cent buffered ethylene glycol. Ethylene glycol was chosen as the solvent, because Geschickter[7] has used it successfully in this capacity in staining tissue cultures. Its antiseptic properties are advantageous in preventing the growth of bacteria in the gel over a long period of time.

METHOD

As the buffer salts (sodium hydroxide and potassium acid phosphate) required to make gels of the desired hydrogen ion concentration are insufficiently soluble in ethylene glycol, some water had to be added. For uniformity, all solutions were made to contain 80 per cent of the glycol, the rest being water and buffer salts as required. This solution was used in the preparation of 5 per cent gelatin gels. The hydrogen ion concentration of sample solutions was measured by Dr. W. C. Harden of Hynson-Wescott-Dunning, Baltimore, Md., with the quinhydrone electrode and colorimetric standards prepared from these solutions were used as checks. Solutions of pH 5.5 and pH 7.65 were used.

The unsolidified gel was divided into two portions. One portion was poured into a 16 mm. test tube until the tube was approximately half full and allowed to solidify in the thermostat at 0° C. To the other portion was added

From the Department of Chemistry, The Johns Hopkins University, Baltimore, Md.
Received for publication, October 22, 1942.

sufficient dye to make a solution M/200 in actual dye content. The gel containing the dye was poured over the solidified clear gel of the same pH and placed in the thermostat. The dye was therefore diffusing into a medium identical in all respects, except for the presence of the dye, with that in which it was dissolved.

In the measurement of the diffusion, a photometer was used to compare the amount of light transmitted through a neutral glass filter and through the gel containing the dye solution. Two glass filters were used corresponding to different concentrations of dye, c_1 and c_2, with c_1 representing a larger concentration of dye than c_2. Daily measurements were made of the distances (d_1 and d_2) from the initial concentration of the dye, M/200, to the concentrations c_1 and c_2 measured by the photometer. In principle, this method is similar to that used by Furth[6] and by Fautrez and Lison[3] who measured the distances the dye diffused to reach a concentration which was a definite fraction of the initial concentration. Their experimental arrangements differ from those described in this paper in that they used microtechnique and measured diffusion in the solvent alone rather than in a gel.

The boundary between colored and colorless gel was also measured. Here the setting was not a photometric one; the line of demarcation between colored and clear gel as intensified by the optical instrument was measured. The symbol d_3 represents the distance of diffusion of the dye from its initial concentration, M/200, to a concentration so small that to visual observation it appears to be zero.

The laboratory measurements of two or more different samples of the same dye were plotted, using as ordinates the distances in mm. and as abscissae the time in days. A smooth curve was drawn between the points and the distances read from this curve. Fig. 1, in which the circles and triangles represent readings from two samples, illustrates the procedure followed and indicates the amount of error involved in the measurement.

RESULTS

In Table I are assembled the distances in mm. which the dyes diffused at the end of 20 days.

TABLE I

DISTANCES OF DIFFUSION IN MM. AFTER 20 DAYS

DYE	pH 5.5			pH 7.65		
	d_1	d_2	d_3	d_1	d_2	d_3
Congo Red—com.	9.2	10.6	12.85	9.45	11.0	12.4
Congo Red—purified	9.0	10.4	12.9	9.5	10.6	12.3
Pontamine Blue BBF	10.05	11.1	12.1	9.5	10.6	11.6
Orange II	13.4	14.6	16.85	14.0	15.6	17.55
Metanil Yellow	13.6	14.9	16.8	14.05	15.85	17.6
Chrysoidine Y	14.75	16.4	18.6	17.4	18.45	19.45
Mercurochrome				13.6	14.4	15.45

Conn[2] has shown that impurities present in the dye may affect its usefulness as a stain. As some of the dyes were technical samples and others carefully purified, it was important to discover whether the presence of impurities commonly found in technical samples would influence diffusion when

the actual dye content of the two samples was the same. The differences in the diffusion of a commercial sample of Congo Red and of a purified sample were found to be negligible, usually 0.2-0.3 mm. or less. This statement is based on a daily comparison of the diffusion distances of duplicate samples of the two dyes over a period of 30 days. Probably the buffer salts in the solution make the effect of additional salts present in the commercial sample negligible. These results are in agreement with the work of Valko.[17]

Table I gives the results of measurements at the two pH values investigated—one of them, pH 5.5, being very near the isoelectric point of gelatin; the other, on the alkaline side. There is little regularity in the slight differences in distance at the two pH values for the dyes with higher molecular weight; the dyes of lower molecular weight, which may be considered in true solution, show a slight but regular tendency towards more rapid diffusion in the solutions of higher pH. Mommsen[10] and Pischinger[12] in studying the effect

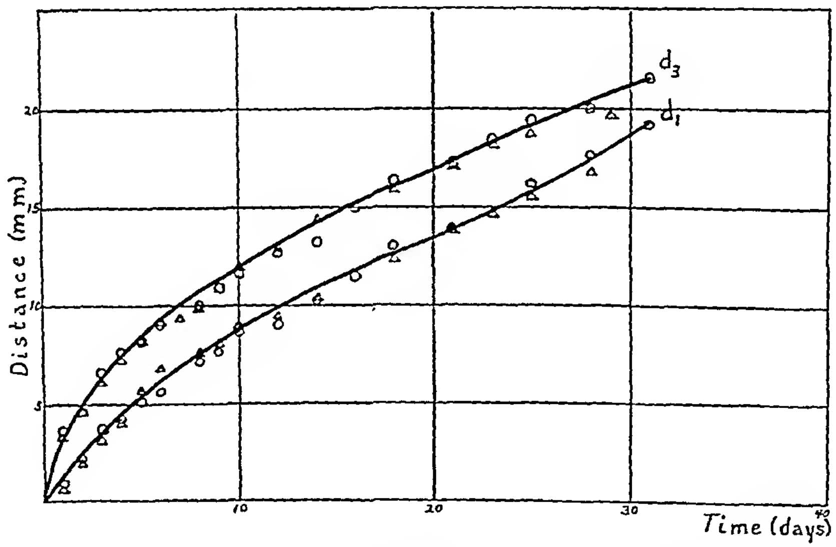


Fig. 1.—Metanil Yellow pH 5.5. Circles and triangles represent measurements on different samples.

of changing pH on the diffusion of dyes in gelatin gels have found that acid dyes diffuse more readily in less acid media and basic dyes more readily in less basic solutions. Their experiments cover a wider range of pH, especially on the acid side; our experiments fall within the pH range in which Pischinger found very slight changes in the diffusion distances.

From Stefan's development of Fick's law, the square of the amount of substance diffusing divided by the time should have a constant value if the initial concentration of the diffusing substance and the cross section in which diffusion is taking place remain unchanged. Stefan found that the same relationship should be true if the amount of substance is replaced by the distance of diffusion to a definite concentration, thus giving a constant value proportional to the diffusion constant. These relations were tested experimentally and found true, at least as a first approximation.[18]

Values of $\frac{d^2}{t}$ were calculated from the three measurements described above. It is evident from Fig. 2, in which values of $\frac{d^2}{t}$ are plotted as ordinates against corresponding values of t as abscissae, that in the case of the first two measurements, d_1 and d_2, constants were not obtained, but that with the third measurement, representing diffusion from a solution M/200 to an in-

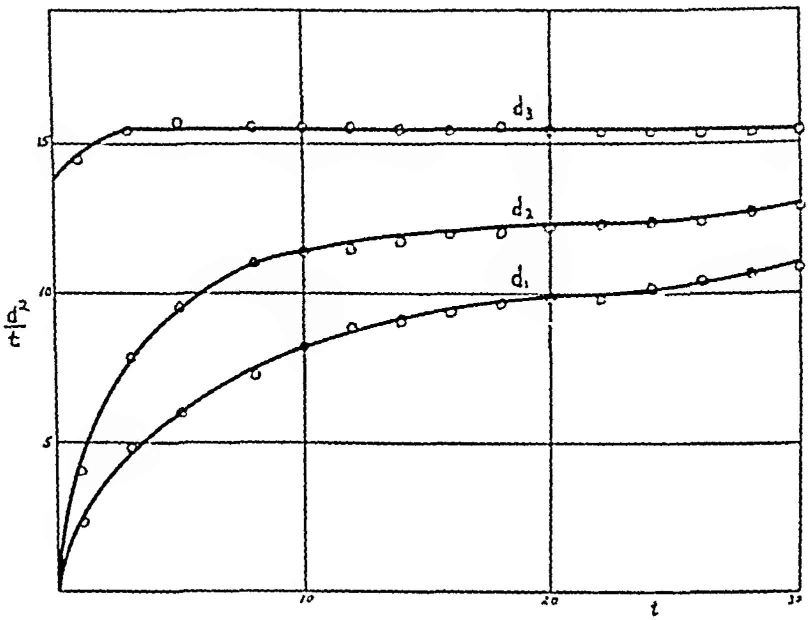


Fig. 2.—Orange II pH 7.65.

finitely dilute concentration of dye, a constant resulted. Further inspection shows that in the first two measurements, values which should be constant increase with increase in time. This suggests a logarithmic curve. Figs. 3 and 4 represent logarithmic curves for diffusion distances of typical dyes. Logarithms of the distances diffused were plotted as ordinates with logarithms of the corresponding times as abscissae. Straight lines were obtained with the first two measurements, d_1 and d_2, as well as with the third measurement, d_3. The slope of the line for d_3 is 2, giving constant values for $\frac{d^2}{t}$, but the slope of the lines for d_1 and d_2 is some fractional value between 1 and 2. When values of $\frac{d^n}{t}$ are calculated for the distances represented by d_1 and d_2, with n representing the slope as determined from the logarithmic curves, constants are obtained. In Table II, values of $\frac{d^2}{t}$ are compared with values of $\frac{d^n}{t}$ for a typical case. In Table III, values of the slope, n, and of the constant, k, from the expression, $k = \frac{d^n}{t}$, are assembled for the various dyes.

DISCUSSION

Many of the logarithmic plots show a different slope at the beginning of the process from that followed during the later stages (see Figs. 3 and 4).

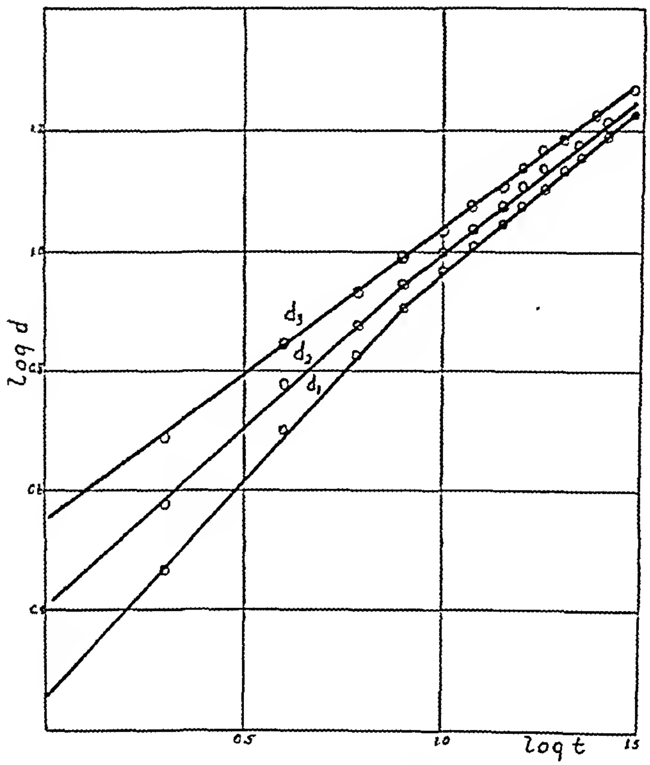


Fig. 3.—Mercurochrome pH 7.65.

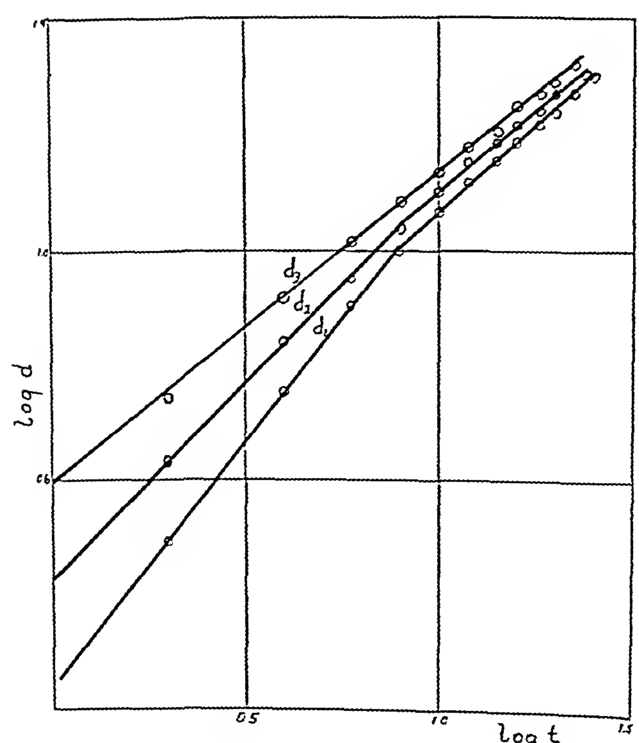


Fig. 4.—Chrysoidine pH 7.65.

This means that the advance to a definite concentration is slower and that n has a lower value in the initial stages of penetration. Similar results have been observed by Stiles[15] and by Bigwood[1] in studies of the penetration of electrolytes and of dyes. Change in structure of the gel with ageing may account for the increase in diffusion. In such cases the values of n in Table III are taken after the change in slope which occurs when diffusion has taken place for 8-10 days.

TABLE II

CALCULATION OF DIFFUSION CONSTANTS

CONGO RED, pH 7.65

n is a constant representing the slope of the logarithmic curves

t	$\frac{d_1^2}{t}$	$\frac{d_1^n}{t}$	$\frac{d_2^2}{t}$	$\frac{d_2^n}{t}$	$\frac{d_3^2}{t}$
3	1.61	1.04	3.41	2.18	7.05
6	2.66	1.23	4.51	2.38	8.05
9	3.24	1.26	4.98	2.29	7.93
12	3.63	1.26	5.33	2.39	7.68
15	3.95	1.26	5.28	2.27	7.63
18	4.30	1.27	5.44	2.21	7.61
21	4.57	1.27	5.66	2.24	7.56
24	4.77	1.26	5.90	2.26	7.71
27	4.98	1.26	5.97	2.23	7.78
30	5.21	1.26	6.16	2.25	7.80
33	5.28	1.24	6.28	2.24	7.80
Average		1.26		2.28	7.69

TABLE III

SUMMARY OF DIFFUSION CONSTANTS OF DYES

FROM THE EQUATION $k = \frac{d^n}{t}$

DYE	d_1 n_1	d_1 k_1	d_2 n_2	d_2 k_2	d_3 n_3	d_3 k_3
pH 5.5						
Congo Red	1.27	0.80	1.446	1.47	2	8.19
Congo Red—com.	1.23	0.73	1.48	1.64	2	8.09
Orange II	1.613	3.29	1.714	4.90	2	13.89
Metanil Yellow	1.456	2.23	1.587	3.62	1.91	10.99
Chrysoidine Y	1.415	2.26	1.617	4.62	2	17.09
pH 7.65						
Congo Red	1.439	1.26	1.613	2.28	2	7.69
Congo Red—com.	1.336	0.96	1.638	2.50	2	7.73
Pontamine Blue BBF	1.754	2.60	1.754	3.18	2	6.72
Mercurochrome	1.765	4.92	1.887	7.56	2	11.82
Orange II	1.626	3.65	1.808	7.16	2	15.43
Metanil Yellow	1.6	3.44	1.719	5.75	2	15.68
Chrysoidine Y	1.746	7.36	1.818	10.01	2	18.95

Even if the conditions upon which Fick's law is based (maintenance of the initial concentration of the diffusing substance and of the cross section in which diffusion is occurring) are observed, deviations have been noticed especially in the diffusion of a colloidal substance within a gel. One explanation[8] which has been offered is that the gel may act as a sieve, holding back larger particles (which may be caused by association of molecules or ions) and permitting the passage of smaller particles. This effect is unlikely in the present

experiments, because deviations are observed with dyes of large and of small molecular weights and because the dye in penetrating into the gel finds the same structure of gel as that in which it was dissolved. Another and more probable cause of the apparent deviations reported in this paper is adsorption of the dye on the fibers of the gelatin. Moravek[11] has measured the penetration of the lead ion into a gelatin gel containing bichromate ion and finds that the results may be expressed by a parabolic equation:

$$a = kt^{\frac{1}{n}}$$

in which a represents the depth of the diffusion layer, t the time, and k and n are constants. He ascribes his results to the affinity of the ion for the gel. It will be seen that the above equation is in agreement with the one we have used.

Friedman[5] has studied the relationship between the molecular weight of nonelectrolytes and their diffusion constants measured in gelatin. When he plots $\frac{1}{\sqrt{M}}$ against k times 10^7, the values fall near a straight line, showing that the size of the molecule influences diffusion to a large extent but that each substance does have some specific effect on the gelatin which prevents the product $k\sqrt{M}$ from being constant. The nonelectrolytes which he investigated had relatively low molecular weights. As the molecular weight and the colloidal nature of the substance increases, it is reasonable that such deviations would become more pronounced. Table IV gives the values $k_3\sqrt{M}$ for the dyes investigated. The constant k_3 was chosen because in this case diffusion was not masked by interfering factors. The symbol M represents the relative weight of the dye ion.

TABLE IV

DYE	M	$\sqrt{M}$	k_3	$k_3\sqrt{M}$
	pH 5.5			
Congo Red	650	25.5	8.19	208.8
Orange II	327	18.08	13.89	251.2
Chrysoidine Y	213	14.6	17.09	249.4
Metanil Yellow	352	18.76	10.99	206.2
	pH 7.65			
Congo Red	650	25.5	7.69	196.1
Pontamine Blue BBF	840	28.98	6.72	194.8
Mercurochrome	707	26.59	11.82	314.3
Orange II	327	18.08	15.43	279.0
Metanil Yellow	352	18.76	15.68	294.1
Chrysoidine Y	213	14.6	18.95	276.6

From a study of the above table one is impressed by the rapid diffusion of mercurochrome. Its molecular weight should indicate slow diffusion and colloidal dispersion. Yet its value for $k_3\sqrt{M}$ shows that it is more closely related to the dyes which are considered to be molecularly dispersed. Teague and Buxton[16] have measured the dialysis of various dyes and find that eosin, which has the same general structure as mercurochrome, can be dialyzed to approximately the same extent as chrysoidine although its molecular weight

is three times as large; congo red, with roughly the same molecular weight as eosin, cannot be dialyzed. These facts are in agreement with the results we have obtained.

SUMMARY

In a dye that is to be used as a stain the rate of diffusion through the tissue is important; in a dye that is depended upon as an antiseptic it is much more so. Obviously sterilization cannot extend beyond the zone of penetration.

Diffusion of dyes in a gel closely duplicates diffusion in tissues. Measurements have been made on the diffusion of various dyes into a 5 per cent gel of 80 per cent ethylene glycol buffered at pH 5.5 and pH 7.65, using a photometer to determine the distance of penetration of definite concentrations of the dye. Differences in diffusion distances of a carefully purified sample and of a commercial sample were found to be negligible when the actual dye content of the two samples was the same. When diffusion was measured to a concentration so small that to visual observation it appeared to be zero, the relationship $k = \frac{d^2}{t}$ was found to give constant values; when diffusion was measured to larger concentrations, constants were obtained for $k = \frac{d^n}{t}$, with n representing some fractional value between 1 and 2. The value of n varied with the dye and with the concentration to which diffusion was progressing; for an individual dye it was usually larger when diffusion to a more dilute concentration was measured. Adsorption of the dye on the fibers of the gelatin is believed to account for the fractional values of n. The rapid diffusion of mercurochrome, in spite of its large molecular weight, is noteworthy.

ACKNOWLEDGMENT

The authors wish to thank Mr. Fletcher B. Holmes and Dr. E. M. Hitch of the E. I. duPont Co., Wilmington, Del., and the late Mr. W. C. Holmes of the Color Laboratory, Washington, D. C., for the samples of dyes.

They also wish to thank Dr. A. H. Pfund of the Department of Physics of the Johns Hopkins University for devising the instrument used in these measurements.

REFERENCES

1. Bigwood, E. J.: Distribution of Ions in Gels, Tr. Faraday Soc. 26: 704, 1930.
2. Conn, H. J.: Influence of Impurities Upon Dyes, Stain Technol. 4: 68, 1929.
3. Fautrez, J., and Lison, L.: Etudes sur la diffusibilité des colorants, Protoplasma 27: 169, 1937.
4. Fautrez, J., and Lison, L.: L'etude physicochemique des colorants dans ses applications biologiques, Protoplasma 33: 116, 1939.
5. Friedman, L.: Diffusion of Non-Electrolytes in Gelatin Gels, J. Am. Chem. Soc. 52: 1305, 1930.
6. Furth, R.: Zur physikalischen Chemie der Farbstoffe. IV. Eine neue Methode zur exakten Bestimmung des Dispersitatsgrades der Farbstofflosungen, Kolloid-Ztschr. 41: 300, 1927.
7. Geschickter, C. F., Walker, E. P., Hjort, A. M., and Moulton, C. H.: A New, Rapid Method for Tissue Diagnosis, Stain Technol. 6: 3, 1931.
8. Herzog, R. O., and Polotzky, A.: Die Diffusion einiger Farbstoffe, Ztschr. f. phys. Chem. 87: 449, 1914.
9. Holmes, W. C.: Mechanism of Staining. The Case for the Physical Theories, Stain Technol. 4: 75, 1929.
10. Mommsen, H.: Ueber den Einfluss der Wasserstoffionen Konzentration auf die Diffusion von Farbstoffen in eine Gelatine gallerte, Biochem. Ztschr. 168: 77, 1926.
11. Moravek, V.: Ueber die Diffusion in Gelen, Kolloid-Ztschr. 49: 39, 1929.

12. Pischinger, A.: Diffusibilitat und Dispersitat von Farbstoffen und ihre Beziehung zur Farbung bei verschiedenen H-ionen-konzentrationen, Ztschr. f. Zellforsch. u. mikr. Anat. 5: 347, 1927.
13. Schulemann, W.: Ueber Vitalfarbung, Ztschr. f. angew. Chemie 34: 237, 1921.
14. Stearn, A. E., and Stearn, E. W.: Mechanism of Staining Explained on a Chemical Basis, Stain Technol. 5: 17, 1930.
15. Stiles, W.: Penetration of Electrolytes Into Gels. 1. The Penetration of Sodium Chloride Into Gels of Agar-Agar Containing Silver Nitrate, Biochem. J. 14: 58, 1920.
16. Teague, O., and Buxton, B. H.: Die Agglutination in physikalischen Hinsicht. IV. Die Ausflockung von Anilinfarben, Ztschr. f. phys. Chem. 60: 479, 1907.
17. Valko, E.: Measurements of the Diffusion of Dyestuffs, Tr. Faraday Soc. 31: 230, 1935.
18. Voigtlander, F.: Ueber die Diffusion in Agargallerte, Ztschr. f. phys. Chem. 3: 316, 1889.

SEASONAL VARIATIONS IN SOME PHYSIOLOGIC VARIABLES*

M. A. WENGER, PH.D., YELLOW SPRINGS, OHIO

THROUGHOUT the psychophysiologic study of the child subjects of the Fels Institute it has become increasingly evident that the season of measurement is an important factor influencing the results. It has been shown[10] that, in general, physiologic responses for these subjects were more consistent over a twelve-month period from winter to winter than they were over a six-month period from winter to summer.

It was thought at first that the variability of the summer data might be the result of the unstable temperature which prevails in summer in the area where the measurements were made. It was discovered, however, that although there tends to be some correlation between environmental temperature in summer and response for certain of the variables measured, some subjects were showing consistent variations which were opposite in direction to that shown in the above correlations. It was postulated that true seasonal variations were demonstrated in our data.

These seasonal variations were illustrated for one variable in an article reporting our method of measuring what has been termed "autonomic balance," i.e., a quantitative estimate of the functional state of the autonomic nervous system.[11, p. 433] The autonomic scores for six children over a period of 18 months were shown. Two cases represented those individuals who have higher scores (indicative of parasympathetic predominance) in the winter than in the summer. A second group of four cases illustrated children who show higher scores in the summer than in the winter. Another set of six cases[11, p. 432] indicated that other children may show no marked variation between the two seasons.

Since that report, additional data have been collected during the summer of 1941 and the winter of 1942. The present paper presents an analysis of all the data for those variables involved in the determination of autonomic balance. Measurements of systolic and diastolic blood pressure have also been studied. Specifically, we wish to know (a) whether there are significant group differences between summer and winter measurements for these physiologic

*From the Samuel S. Fels Research Institute, Antioch College, Yellow Springs, Ohio.
Received for publication, October 22, 1942.

responses, and (b) whether individuals show consistent differences in direction of seasonal variation.

No extensive reference is made to the literature related to these problems. Earlier work on human subjects has, in most instances, involved two few cases to answer the first question. The controversinl results reported on a few cases for such variables as heart rate,[2,4] respiration rate,[5] and others,[1,4,5] may be construed as evidence that the second problem proposed above is deserving of study. Certainly the studies of Peterson,[7] Mills,[8] and Pottenger[9] attest to the importance of the weather as a factor in health and disease, and therefore in general physiologic function.

The data reported herein have been collected in a routine manner by standardized procedures already described.[10] During the winter months of January, February, and March, all of the child subjects of the Institute who are six years of age or older come to the laboratories for a full day of measurement and study. During the summer months of June, July, and August, they again visit the laboratories for periods varying from one day to two weeks. All physiologic measurements are conducted during the hours between 9 A.M. and 12 noon. All but two, salivary output and standing palmar skin conductance, are measured in the resting state. Whenever possible, the recorded datum for an individual represents the mean of a series of samples during the examination period, which lasts about forty-five minutes. The temperature of the measurement room is maintained at 74 to 76° F. during both summer and winter, and the relative humidity is maintained at approximately 40 per cent during the winter and 60 per cent during the summer. For the purposes of this paper this difference in relative humidity is regarded as being a component of the season of measurement.

THE GROUP ANALYSIS

Since the majority of the variables studied bear some relationship to age, it was necessary to eliminate this factor from the analysis. This was done by the simple technique of comparing the means of the data for two (then three) successive winters with the values for the intervening summer (then the mean of two successive summers). The midpoint for chronological age is thus identical for the groups of data compared in each analysis. The first part of Table I shows the means, standard deviations, reliability coefficients, and criteria for judging the significance of the differences between the means for the winters of 1940 and 1941 and the summer of 1940. The data are the results of ten physiologic measurements of fifty-one children ranging in age from 6 to 12 years. The second half of the table shows a similar analysis for twenty-three of those subjects for whom data were available for the summer of 1942. Here, since a smaller number of cases was involved, the standard error of the difference has been based upon the standard deviation of the differences themselves as demonstrated by each subject.*

*The formula for the standard error of the difference between means used in the first analysis is that illustrated by Peters and Van Voorhis[11] as No. 92. For the second analysis, see their formula No. 95. The correlations employed in the first analysis are those obtained for the winter and summer data of 1940. In the second analysis (23 cases) the 1942 winter data for diastolic blood pressure and palmar skin conductance were not included, because part of them were believed to be unreliable. Since one influenced the scores of pulse pressure and both affected the derived scores of autonomic balance, the 1942 winter data for these variables also were omitted from the analysis. Diastolic blood pressure, the only one of these four variables significantly related to chronological age, was corrected for the resulting age discrepancy.

TABLE I

SEASONAL DIFFERENCES BETWEEN MEANS WITH t AND P VALUES OF THE DIFFERENCES FOR TEN PHYSIOLOGIC MEASUREMENTS OF CHILDREN AGED 6 TO 12 YEARS

	N = 51									N = 23							
NO. AND VARIABLES	SUMMER 1940		WINTERS 1940 AND 1941		DIFFERENCE				SUMMERS 1940 AND 1941		WINTERS 1940, 1941 1942	M DIFFERENCES				
	M_1	σ	M_2	σ	R	M_1-M_2	σM_1-M_2	t	P	M_3	M_4	3-4	σD	σM_3-M_4	t	P
23 Dermographia Persistence (minutes)	12.20	9.02	12.67	10.95	.41	-0.47	1.54	0.31	.76	11.78	12.57	-0.79	9.21	1.96	0.40	.69
25 Salivary Output (c.c. per 5 minutes)	4.41	2.46	4.06	2.04	.70	0.35	.25	1.40	.16	4.83	4.57	.26	1.26	.27	0.97	.35
28 Heart Period (seconds)	.745	.05	.723	.086	.66	.022	.009	2.44	.01	.757	.740	.017	.045	.01	1.70	.10
37 Palmar Conductance (microhms)	23.3	6.8	33.5	11.0	.32	-10.2	1.53	6.67	<.01	29.0	33.8	-4.8	9.42	2.01	2.39	.03
39 Volar Conductance	14.2	8.5	14.2	6.3	.12	0.0	1.39	0.0		11.4	13.3	-1.9	5.41	1.15	1.65	.12
57 Respiration Period (seconds)	3.01	1.95	3.19	1.04	.37	-0.18	.26	0.69	.49	2.99	3.18	-0.19	.518	.110	1.73	.10
76 Systolic Blood Pressure (mm. Hg)	96.20	6.40	98.50	5.99	.70	-2.30	.67	3.43	<.01	96.48	99.48	-3.00	3.47	.740	4.05	<.01
77 Diastolic Blood Pressure	60.70	6.74	61.60	7.45	.55	-0.90	.95	0.95	.34	59.96	60.35	-0.39	5.59	1.19	0.33	.72
80 Pulse Pressure	35.50	5.96	36.50	8.26	.38	-1.00	1.14	0.88	.38	35.17	37.30	-2.13	5.20	1.11	1.92	.07
A Autonomic Balance	72.50	9.03	72.65	7.97	.57	-0.15	1.11	0.14	.88	50.78	51.65	-0.87	6.17	1.32	0.66	.52

It will be seen that both analyses agree in placing the seasonal difference in systolic blood pressure at better than the .01 level of significance. The probability is almost as remote that the difference in palmar skin conductance could have occurred by chance; and the chances are high that the seasonal change in heart period also represents a true difference. Although the level of significance for none of the other measures is as high in either analysis as .05, it is of interest that both analyses agree in the direction of the differences.

From these results the first question may be answered. The probability is great that during the summer, as compared with the winter, systolic blood pressure is lower, standing palmar skin conductance (palmar sweating) is lower, and the heart beats at a slower rate. There also is some indication that salivary output is greater, respiration rate is faster, and pulse pressure is lower. The chances are low that true differences obtain for diastolic blood pressure, volar skin conductance, dermographia persistence, or autonomic balance, although the differences found here for these four variables are in the same direction in both analyses. The small change in the estimates of autonomic balance is of particular interest, as will be made clear in the following discussion.

For convenience in interpreting the results the 1940 regression equation[12] for the estimation of autonomic function is reproduced below. This equation is used because it is believed to be more representative of autonomic function than the 1941 equation, scores on which are greatly influenced by individual deviations in heart period.

$$\overline{Nu} = .15z_{23} + .39z_{25} + .31z_{28} + .38z_{37x} + .10z_{39x} - .10z_{5} + .22z_{6}$$

In the equation, $\overline{Nu}$ signifies the estimate of autonomic balance for a given individual; z equals the individual's standard score in the trait designated by the number in subscript; and x, following trait numbers 37 and 39, indicates that these traits have been reflected so that a high standard score is the equivalent of a low raw score. The names of the traits are the same as shown in Table I. A high score derived by means of this equation is considered indicative of functional predominance of the parasympathetic branch. A homogeneous shift in all functions toward the parasympathetic end of the scale would involve a lengthened persistence of red dermographia, an increased salivary output, a slowed heart rate, decreased palmar and volar conductance or sweating, an increased respiration rate, and a heightened pulse pressure. With the exception of dermographia persistence and pulse pressure, this is the pattern of response for summer (shown in Table I). Since these two variables are not heavily weighted in the equation, it might be expected that seasonal comparisons of these scores would reveal a significant difference between summer and winter scores of autonomic balance, with the summer season effecting a shift toward the parasympathetic end of the scale. Such a shift might also be predicted from the findings of Gellhorn and Feldman,[3] who report that heat, although affecting both the vago-insulin and sympathetico-adrenal systems, has its greatest effect upon the former. Table I shows, however, that no reliable difference obtains. The next section of this report will deal further with this finding.

THE PROFILES FOR 23 INDIVIDUALS

The individual data for the 23 children who had had five consecutive measurements were analyzed in the same general manner as were the group data for 51 cases. The scores for a given subject were combined in three ways, two combinations being the mean of two adjacent winters and the intervening summer, and the third being the mean of the two summers and the intervening winter. If the summer score for a given variable was higher than the winter score *for all three combinations,* the child was scored plus (+) for that variable. If the winter score was higher than the summer score *for all three combinations,* the child was scored minus (−). If the combinations differed in direction of change, no symbol was assigned. Instances of "no change" were infrequent and, when occurring among otherwise consistent changes, were disregarded. The series of 23 profiles obtained in this manner are shown in Table II. It should be noted that four of the variables have been reflected to make their direction consistent with respect to autonomic activity.

It will be seen that the cases are grouped in four categories. Since systolic blood pressure and palmar skin conductance had shown the most significant seasonal variation, it was argued that consistent variation in either of these variables in a direction opposite to that shown by the group might be considered as of possible significance. As might be expected, the largest group, comprising nine cases, is that which shows during the summer measurements a lower systolic blood pressure *and* a lower palmar conductance. Group II contains the five subjects who manifest higher systolic blood pressure in summer. Group III comprises four children who show higher palmar conductance in summer than in winter. Group IV includes five cases concerning which the criterion data are indecisive. The criterion data are underlined in Table II.

It is of interest to note that heart rate is slower in summer for Group I, faster in summer for Group II, and inconsistent in change for Group III. Pulse pressure also shows a certain differential group consistency. In general, it appears to be decreased in summer in Group I and increased in Groups II and III. Diastolic pressure tends to show the opposite picture. Although the results are not decisive, dermographia persistence time seems shorter in summer for Groups II and III, while volar skin conductance is lower in summer for Group I. The groups are not well differentiated for the variables salivary output or respiration period.

The first seven variables of Table II are those used in the estimation of "autonomic balance." With the reflection of the skin conductance variables 37 and 39, positive signs for all variables indicate a shift toward greater parasympathetic function; conversely, negative signs indicate a shift toward functional predominance of the sympathetic system. While there is some suggestion that Group I shows a majority of positive symbols, and Groups II and III, a majority of negative signs, a more meaningful interpretation may be drawn from the last row of the table showing the direction in shift of the derived autonomic scores. Here Groups II and III are seen to contain no positive signs. Group I contains a majority of positive signs in the last row, and the data offer a ready explanation for the three negative symbols, the encircled symbols for

TABLE II

PROFILES FOR 23 CHILDREN SHOWING THE DIRECTION OF CONSISTENT SEASONAL VARIATIONS IN TEN PHYSIOLOGIC VARIABLES OVER THREE WINTERS AND TWO INTERVENING SUMMERS*

NO. AND VARIABLE		GROUP I									GROUP II					GROUP III				GROUP IV				
CASE NO. AND SEX		3F	4M	17F	75F	77M	87M	88F	91F	105F	20M	73F	81M	83F	86F	28F	74M	82F	92M	41F	42F	67F	85M	102M
23	Dermographia Persistence (minutes)	+		–	–			–	+	+				–	–	+	–	–	–	–		–	+	
25	Salivary Output (c.c. per 5 min.)		⊖	+	⊖	–	+	+		–	–	–	–				+	+				+		–
28	Heart Period (seconds)	+	+	+	+		+	⊖	+	+	–	–		–	–						–			+
37x	Palmar Conductance (microhms)	+	+	+	+	+	+	+	+	+	+	+		+	+	–	–	–	–	+	+	+		+
39x	Volar Conductance	+	–	+			+	+	+	+	–		–	+			+		–		+	+		
57	Respiration Period (seconds)	+					–	–		–										–	–	–		+
80	Pulse Pressure		–	–			–	–	+	–		+	+	+	+		+	+				–	+	–
76x	Systolic Blood Pressure (mm. Hg)	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+					
77x	Diastolic Blood Pressure	+				+		–	+	–	–	+	+	+	+		+	+		–		–		–
A	Autonomic Balance	+	–	+	–	+	+	–	+	+	–	0	–	–	–	–	–	–	–	–	–	+	+	–

* + signifies consistently higher scores in summer than in winter.
– signifies consistently lower scores in summer than in winter.
x indicates reflection of variable.

the heavily weighted variables of salivary output and heart rate. Perhaps these encircled symbols represent experimental artifacts or discrete functional differences apart from the total variance in autonomic function.

In terms of this latter method of differentiation these subjects might be reclassified into two groups. Six cases from Group I and two from the indeterminate Group IV comprise those individuals who show a relatively greater parasympathetic function in summer than in winter (Group +). Groups II and III and the remainder of the cases of Groups I and IV are those who manifest relatively greater sympathetic function in summer (Group −). Since the two groups are rather evenly divided, the explanation for the lack of a reliable total group seasonal variation becomes apparent. It is of further interest that the two groups differ significantly in mean winter autonomic score. The mean winter autonomic score for Group + is 69.4. The mean for Group − (omitting the one subject who shows no variation from summer to winter) is 77.5, or approximately one σ higher. Since the σ of the difference in means is only 3.14, the obtained t of 2.58 may be considered as significant at approximately the 0.04 level of probability, when only 7 degrees of freedom are allowed. Apparently the summer season tends to effect a shift toward greater parasympathetic activity in those individuals having low autonomic scores (apparent predominance of sympathetic function) in winter, and a shift toward greater sympathetic activity in those individuals demonstrating high autonomic scores (apparent predominance of parasympathetic function) in winter. The summer seasons seem, then, to have a levening effect upon autonomic function, and it seems pertinent to question whether summer might not afford more "basal" conditions for the determination of scores of autonomic balance. Scores obtained in winter might then be viewed as reflecting the reaction of the organism to a prolonged polar front. In most temperate latitudes, however, winter must remain the season of choice for such determinations, for the environmental temperatures during the summer months are not sufficiently stable to permit the collection of comparable data on a large group.

It remains to be determined whether or not this differential seasonal variation is significant in behavior and disease. The results certainly are consistent with the observations of Peterson,[8] Mills,[6] and Pottenger.[9] Furthermore, the different groupings of seasonal variations in different functions serve to re-emphasize a consideration that Peterson has stressed; namely, the necessity for considering individual differences in response to climatic changes in any study of physiologic function.

SUMMARY

This study has concerned itself with two questions: (a) Are there significant group differences between summer and winter measurements of certain physiologic responses. (b) Do individuals show consistent differences in direction of seasonal variations. Two analyses of group data have been made; one involving data obtained during two consecutive winters and the intervening summer for 51 children 6 to 12 years of age, and the other involving data obtained during an additional winter and summer for 23 of the same children. Individual profiles were shown for these 23 cases.

The two group analyses agree in finding systolic blood pressure and palmar skin conductance (palmar sweating) significantly lower during the summer measurements. The chances are high that heart period also is significantly longer in summer. Although none of the other differences found may be regarded as reliable, there is some indication that in summer as compared with winter, diastolic blood pressure and pulse pressure are lower, salivary output is greater, respiration is faster, dermographia persistence time is shorter, and volar conductance or sweating is decreased.

The analysis of individual profiles shows that the seasons consistently have different effects on different individuals for each of the variables studied. It is believed that individuals may be meaningfully classified in at least two groups in terms of scores of autonomic balance: those who manifest relatively greater parasympathetic function in summer than in winter, and those who demonstrate relatively greater sympathetic function in summer than in winter.

REFERENCES

1. Apperly, Frank L.: Variations in Pulmonary Vital Capacity in Health: Daily, Seasonal, and at Moderate Altitudes, Proc. Soc. Exper. Biol. & Med. 40: 291, 1939.
2. Boas, E. P., and Goldschmidt, E. F.: The Heart Rate, Springfield, Illinois, 1932, Charles C Thomas, page 166.
3. Gellhorn, E., and Feldman, J.: The Influence of Cold and Heat on the Vago-insulin and the Sympathetico-Adrenal Systems, Am. J. Physiol. 133: 670, 1941.
4. Griffith, Fred R., Jr., Pucher, Geo. W., Brownell, Katherine A., Klein, Jennie D., and Carmer, Mabel E.: Studies in Human Physiology II. Pulse Rate and Blood Pressure, Am. J. Physiol. 88: 295, 1929.
5. —: Studies in Human Physiology IV. Vital Capacity, Respiratory Rate and Volume, and Composition of the Expired Air, Am. J. Physiol. 89: 555, 1929.
6. Mills, C. A.: Medical Climatology, Springfield, Illinois, 1939, Charles C Thomas, page 300.
7. Peters, C. C., and Van Voorhis, W. R.: Statistical Procedures and Their Mathematical Bases, New York, 1940, McGraw-Hill Book Co., page 516.
8. Peterson, Wm. F.: The Patient and the Weather, Ann Arbor, Michigan, 1934-38, Edwards Bros., Vol. I-IV.
9. Pottenger, F. M.: Living With the Weather, Ann. Int. Med. 14: 502, 1940.
10. Wenger, M. A.: A Study of Physiological Factors: The Autonomic Nervous System and the Skeletal Musculature, Human Biol. 14: 69, 1942.
11. —: The Measurement of Individual Differences in Autonomic Balance, Psychosom. Med. 3: 427-434, 1941.
12. —: The Stability of Measurement of Autonomic Balance, Psychosom. Med. 4: 94, 1942

THE EFFECT OF CONTINUED SULFANILAMIDE INGESTION ON THE ACID-BASE EQUILIBRIUM OF THE DOG*

ALFRED H. FREE, PH.D., DONALD E. BOWMAN, PH.D., AND DEAN F. DAVIES, M.S.
CLEVELAND, OHIO

A NUMBER of studies have been made of the effect of sulfanilamide on the acid-base equilibrium of man and animals.[2-10] Some of these studies have been carried out in patients in whom complicating effects were present. Others have either employed quantities of sulfonamide outside the range that is considered therapeutically effective or have been confined to observations following single doses of the drug. The general results of these studies have indicated that in some patients and animals evidence of acidosis is present following sulfanilamide ingestion. As a result of the effects that arise from sulfanilamide, the use of alkali with the compound has been suggested and has been rather widely used.

The present report describes studies conducted with normal dogs which were given sulfanilamide either alone or accompanied by sodium citrate. The purposes of these studies were: (1) to observe the changes in the acid-base equilibrium of the blood following the ingestion of quantities of sulfanilamide sufficient to maintain therapeutic levels in the blood; (2) to determine the changes in the acid-base equilibrium of the blood produced by combinations of sodium citrate and sulfanilamide; (3) to ascertain whether the restriction of dietary salt intake would influence the effects of sulfanilamide with or without alkali; (4) to observe the effect on the acid-base equilibrium of the blood when the concentrations of sulfanilamide are maintained above therapeutic levels over a period of several days; (5) to determine the effect of single massive doses of sulfanilamide; (6) to examine the changes in urine pH during the maintenance of high levels of blood sulfanilamide.

METHODS

Adult female dogs were employed in all of the studies and were maintained on a diet of Purina dog chow except during the studies with the low salt diet. This low salt diet was similar to that employed by Nilson,[11] consisting of cracker meal, cane sugar, casein, butterfat, and lard, and having a very low content of sodium, potassium, and chlorine. Blood samples were obtained from the jugular veins with a minimum of stasis, and urine samples were obtained by catheteriza-

*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

Presented before the Federation of American Societies for Experimental Biology,[1] at Chicago, April, 1941.

Received for publication, Dec. 23, 1942.

tion. Methods of handling the blood as well as the references to the chemical methods employed are reported elsewhere.[16] Hemoglobin determinations were carried out on whole blood by the method of Evelyn,[17] while red and white cell counts were made by conventional hematologic techniques.

CHANGES OBSERVED IN THE ACID-BASE BALANCE OF THE BLOOD

Animals Receiving a Normal Diet.—In this series of experiments the dogs were given sulfanilamide alone, sulfanilamide and sodium citrate, or sodium citrate alone while ingesting a normal diet. Three dogs were included in each group and, by shifting the animals, the same three dogs acted as subjects in each group. Approximately ten to twelve days elapsed between the end of one experiment and the beginning of the next. The routine used in these experiments was as follows: A fasting blood sample was obtained at 8:30 A.M. on the first day of the experiment, and this was subjected to a "complete analysis" which included determinations of serum pH, carbon dioxide content, chloride, inorganic phosphorus, protein, and total base and whole blood determinations of hemoglobin, sulfanilamide, and red and white cell counts. A sample of urine, obtained by catheterization, was also taken at this time and its pH determined. At 9:00 A.M. each dog received by stomach tube the indicated compound or mixture in an aqueous solution of approximately 500 c.c. The initial dose was twice as large as all the succeeding doses. For this reason the intake of the compounds in question is 33 per cent higher on the first day than on all the following days. A second dose of the drug was administered at 1:00 P.M. and a third dose, at 5:00 P.M. The total daily dose (on all except the first day) of sulfanilamide was 0.20 Gm. per kilogram of body weight, and the dose of sodium citrate was 1.0 Gm. per kilogram. Each single dose represented one third of the daily dose. An afternoon sample of blood was taken at 4:00 P.M. and submitted to "partial analysis," consisting of determinations of serum pH, carbon dioxode content, chloride, and blood sulfanilamide. An afternoon (4:00 P.M.) urine specimen was obtained by catheterization and the pH determined. On the succeeding days of the experiment the same schedule was followed and a "partial analysis" was made on the morning and afternoon blood. Cell counts and hemoglobin estimations were done each morning, and morning and afternoon urine samples were obtained by catheterization for pH estimations. On the morning of the day that the drug was discontinued a sample of blood for "complete analysis" was again obtained.

The results of this experiment are presented in the first four columns of Table I. By the nature of the experiment, the morning analysis represents the blood level at a time fifteen hours after the preceding dose of drug, whereas the afternoon analysis represents the blood level three hours after the preceding dose. During the experiment no trend of change from day to day was discernible, and consequently only the average values for the morning and afternoon samples during the experimental period are recorded. From the data presented in Table I it will be seen that considerable fluctuation in blood sulfanilamide level occurred between the morning and afternoon samples as was to be expected. On the other hand, the level of blood sulfanilamide was approximately the same after sulfanilamide and sodium citrate as after sulfanila-

mide alone. It will be seen that the serum pH was not appreciably altered from the control level by sulfanilamide, by sulfanilamide and sodium citrate, or by sodium citrate alone, and, furthermore, there were essentially no differences in the morning and afternoon samples. The serum chloride was maintained at approximately control levels in all of the present experiments. Serum bicarbonate was slightly decreased after sulfanilamide alone, but in the afternoon samples, three hours after the administration of sulfanilamide and sodium citrate or sodium citrate alone, the serum bicarbonate was increased. The hemoglobin content of the blood was not significantly altered in any of the experiments, the slight variations encountered probably being explained by the loss of the blood removed for the chemical analyses. The erythrocyte and leucocyte counts of the blood were not appreciably changed. No significant changes in total base, protein, phosphate, or undetermined acid were noted between the beginning and end of the experiment.

Animals Receiving a Low Salt Diet.—In this series of experiments, dogs were maintained on a synthetic diet quite low in chloride and total base and were given the same combinations of sulfanilamide and sodium citrate that were employed in the preceding experiments. The low salt diet was given for one week before the start of the study and was continued during the interval of eight days between experiments. Each of the three dogs under observation was shifted so that each served in each group. The routine followed in these experiments was exactly the same as that used in the above studies on the normal salt diet with the exception that no blood or urine was taken on the third and fifth days, although the administration of the drugs was the same on these days. The results of this experiment are indicated in the last four columns of Table I. It will be seen that, in general, the results of this experiment are similar to those obtained when the animals received a diet with a normal salt content. However, throughout the experiment there was a tendency to slightly greater changes in pH and bicarbonate as a result of the ingestion of sodium citrate either alone or with sulfanilamide.

Animals Receiving Sulfanilamide at Four-Hour Intervals.—In order to ascertain the effects of blood concentrations maintained somewhat above the therapeutic levels, and also in order to decrease fluctuations of blood sulfanilamide concentrations, sulfanilamide was administered six times per day at four-hour intervals. The drug was given at 9:00 A.M., 1:00 P.M., 5:00 P.M., 9:00 P.M., 1:00 A.M., and 5:00 A.M. in amounts of 0.05 Gm. per kilogram of body weight per dose. Blood samples were obtained twice each day at 8:00 A.M and 4:00 P.M. Urine samples were obtained at four-hour intervals (see below). Three dogs were studied in this way, the sulfanilamide being given for four, six, and seven days to the three animals. The analyses included blood sulfanilamide and serum pH, bicarbonate, and chloride determinations. The results of these studies are summarized in Table II. Dogs 1 and 3 did not exhibit any adverse effects from this experiment, whereas Dog 2, after the first day of the experiment, completely refused all food and vomited considerably during the period of sulfanilamide ingestion. It will be noted that the blood sulfanilamide concentration in this dog reached higher levels than in the other two dogs and a progressive increase occurred during the latter part of the experiment. At the

TABLE I

AVERAGE BLOOD FINDINGS* DURING CONTINUED ADMINISTRATION OF SULFANILAMIDE AND ALKALI

	NORMAL DIET				LOW SALT DIET			
	CONTROL	DURING ADMINISTRATION OF SULFANILAMIDE†	DURING ADMINISTRATION OF SULFANILAMIDE AND SODIUM CITRATE‡	DURING ADMINISTRATION OF SODIUM CITRATE§	CONTROL	DURING ADMINISTRATION OF SULFANILAMIDE†	DURING ADMINISTRATION OF SULFANILAMIDE AND SODIUM CITRATE‡	DURING ADMINISTRATION OF SODIUM CITRATE§
Blood sulfanilamide A.M. (mg. per 100 c.c.)	0.0	7.9	8.6	0.0	0.0	8.3	7.6	0.0
Blood sulfanilamide P.M. (mg. per 100 c.c.)	0.0	15.8	17.1	0.0	0.4	15.5	13.4	0.0
Serum pH (A.M.)	7.40	7.40	7.42	7.40	7.38	7.39	7.41	7.41
Serum pH (P.M.)	7.41	7.41	7.43	7.42	7.38	7.41	7.43	7.41
Serum chloride A.M. (meq. per L.)	109.2	110.8	110.3	106.5	109.3	107.9	105.8	105.0
Serum chloride P.M. (meq. per L.)	109.2	110.2	108.5	105.3	109.3	107.3	102.6	102.1
Serum bicarbonate A.M. (meq. per L.)	24.1	22.2	24.9	25.0	21.3	21.9	25.2	26.5
Serum bicarbonate (P.M.) (meq. per L.)	24.1	22.3	27.0	29.0	21.3	22.3	28.7	31.5
Hemoglobin (Gm. per 100 c.c.)	12.4	11.3	11.7	12.4	13.5	13.4	12.3	13.4
Erythrocyte count (million per cubic millimeter)	5.7	5.3	5.3	5.5	5.8	5.5	5.6	5.9
Leucocyte count (thousand per cubic millimeter)	8.7	10.1	9.9	11.0	11.0	11.5	12.4	11.9

*Blood taken for analysis twice daily, at 8:00 A.M. and 1:00 P.M.

†0.20 Gm. of sulfanilamide per kilogram of body weight per day, administered in three doses at 9:00 A.M., 1:00 P.M., and 5:00 P.M. each day for a period of six days to three dogs.

‡0.20 Gm. of sulfanilamide per kilogram per day plus 1.0 Gm. of sodium citrate per kilogram per day administered in three doses at 9:00 A.M., 1:00 P.M. and 5:00 P.M. each day for a period of six days to 3 dogs.

§1.0 Gm. of sodium citrate per kilogram per day administered in three doses at 9:00 A.M., 1:00 P.M., and 5:00 P.M. each day for a period of six days to three dogs.

same time the serum chloride in this animal fell from an average control value of 110.3 meq. per liter to a minimum of 83.0 meq. the last day of the experiment. At this time the total base concentration of the serum showed a deficit of the same magnitude as the chloride decrease. However, even though this animal was severely dehydrated, there were no significant changes in the serum pH or the serum bicarbonate concentration. It will be seen that some decrease in serum pH occurred in all three dogs, although the decrease was not progressive. Dog 3 showed a somewhat greater lowering of serum pH than the other two dogs and had a minimum value of 7.32 in one of the samples. The serum bicarbonate content showed some fall in all three animals, although the change was not marked in any.

TABLE II

BLOOD STUDIES IN DOGS RECEIVING SULFANILAMIDE AT FOUR-HOUR INTERVALS

	DOG 1	DOG 2	DOG 3
Average blood sulfanilamide (mg. per 100 c.c.)	30.4	47.5	21.4
Maximum blood sulfanilamide (mg. per 100 c.c.)	43.5	70.8	33.1
Control serum pH	7.40	7.40	7.41
Average experimental* serum pH	7.39	7.37	7.35
Minimum experimental* serum pH	7.35	7.35	7.32
Control serum chloride (meq. per L.)	110.2	110.3	112.0
Average experimental* serum chloride (meq. per L.)	112.4	99.8	110.0
Minimum experimental* serum chloride (meq. per L.)	109.0	83.0	107.0
Control serum bicarbonate (meq. per L.)	25.7	24.7	22.5
Average experimental serum bicarbonate (meq. per L.)	21.3	21.4	20.6
Minimum experimental serum bicarbonate (meq. per L.)	19.3	19.6	19.1

*During sulfanilamide administration.

Animals Receiving a Single Large Dose of Sulfanilamide.—In order to ascertain the effect of large quantities of sulfanilamide on the acid-base equilibrium, dogs were given a single large dose of 1.0 Gm. of sulfanilamide per kilogram of body weight. The sulfanilamide was mixed with approximately 500 c.c. of warm water and was given as an aqueous suspension. With this quantity of sulfanilamide, marked dyspnea developed in all three animals, accompanied by clonic convulsions, spasticity, extreme salivation, and loss of most reflexes. A control blood sample was obtained along with samples at one, three, six, twelve, twenty-four, and forty-eight hours after the administration of the drug. Chemical analyses of the blood included those determinations recorded in Table III. It will be observed that a maximum concentration of blood sulfanilamide was obtained in the three-hour sample, although the concentration in the six-hour sample was quite similar. The values presented in Table III indicate that there were no marked changes in the acid-base equilibrium of the blood, although all of the animals showed marked dysfunction of the central nervous system. Hemoglobin and serum protein concentrations showed elevations that were a maximum at six hours, which would indicate that there was a loss of water from the blood.

CHANGES OBSERVED IN THE URINE pH

In both the experiments with a normal diet and with the low salt diet, it was found that the pH of urine samples obtained by catheterization were acid in the morning and alkaline in the afternoon. It has been suggested that sulfanilamide has some specific effect in preventing bicarbonate reabsorption in the

kidney tubules and that this is the basis for the disturbance in the acid-base equilibrium which follows the ingestion of sulfanilamide. In the experiments with the normal diet and the low salt diet, the blood level of sulfanilamide was at the low point when the urine was acid and was considerably higher when the urine was alkaline. The urine pH was studied more closely in the animals receiving sulfanilamide at four-hour intervals. The routine followed in these experiments has been described above, and it is to be noted that urine samples were obtained by catheterization at four-hour intervals during the day and night. The urine was collected just prior to sulfanilamide administration. With two or three exceptions, the dogs did not urinate between the four-hour catheterizations, so the entire urine output was collected in this manner. The maximum and minimum values of urine pH for the three animals for each of the days that they were receiving sulfanilamide are given in Table IV. It will be observed that a considerable range of urinary pH was encountered during the

TABLE III

BLOOD STUDIES FOLLOWING A SINGLE LARGE DOSE* OF SULFANILAMIDE
AVERAGE RESULTS OBTAINED WITH THREE DOGS

	CONTROL	1 HR.	3 HR.	6 HR.	12 HR.	24 HR.	48 HR.
Blood sulfanilamide (mg. per 100 c.c.)		57.8	67.0	62.0	37.7	18.7	
Serum pH	7.39	7.38	7.37	7.37	7.36	7.36	7.30
Serum total base (meq. per L.)	160.2	157.9	160.2	164.1	165.7	155.8	
Serum chloride (meq. per L.)	110.8	107.7	108.7	110.0	108.5	109.7	108.6
Serum bicarbonate	22.8	21.4	19.6	20.2	19.1	19.4	20.4
B Pr,† (meq. per L.)	14.1	14.8	15.3	16.8	16.5	15.2	
B PO_4‡ (meq. per L.)	4.1	4.3	3.8	3.2	5.6	4.1	
Undetermined acid (meq. per L.)	8.4	9.7	12.8	13.9	16.0	7.4	
Hemoglobin (Gm. per 100 c.c.)	12.3	12.8	15.2	16.0	13.7	12.0	
Urine pH	7.4	7.4	7.8	7.9	7.6	7.0	7.0

*1.0 Gm. of sulfanilamide per kilogram of body weight.
†Base combined with protein.
‡Base combined with phosphate.

TABLE IV

VARIATION IN URINE PH IN DOGS RECEIVING SULFANILAMIDE

EXPERIMENTAL DAY		DOG 1	DOG 2	DOG 3
1	Max.	8.4	7.6	7.0
	Min.	7.0	5.9	5.5
2	Max.	7.8	8.1	7.6
	Min.	6.5	5.9	6.2
3	Max.	7.8	7.4	7.5
	Min.	6.6	5.5	6.1
4	Max.	7.6	7.3	7.6
	Min.	6.6	5.2	5.6
5	Max.	7.5	7.4	
	Min.	5.9	6.9	
6	Max.	7.4	7.2	
	Min.	6.1	6.1	
7	Max.		7.2	
	Min.		6.2	

same day in all of the dogs. With but two exceptions, the maximum pH was on the alkaline side of neutrality while the minimum pH was on the acid side of neutrality. The two exceptions mentioned occurred in Dogs 1 and 3 on the first experimental day. Dog 1 on this day had a maximum pH of 8.4 and a minimum pH of 7.0, whereas Dog 3 had a maximum pH of 7.0 and a minimum pH of 5.5. The only instance in which the fluctuation in pH was less than 1.0 occurred in Dog 2 on the fifth experimental day, when the maximum pH value was 7.4 and the minimum value was 6.9.

DISCUSSION

The present studies indicate that, with continued sulfanilamide administration and the maintenance of therapeutic levels of sulfanilamide in the blood, there are no great changes in the acid-base equilibrium. The small decrease in serum bicarbonate that occurs can be prevented by the administration of sodium citrate along with the sulfanilamide. Hematologic studies in the dogs receiving either sulfanilamide alone or sulfanilamide plus sodium citrate indicate that there was no anemia or leucopenia in any of the animals.

Hartmann[6] has suggested that respiratory alkalosis develops following sulfanilamide therapy and that administration of alkali is undesirable since it would tend to accentuate the alkalosis. Hartmann furthermore suggested that chloride deprivation would result in even more marked changes when alkali was given with the sulfanilamide. The present findings in the dogs maintained on a low salt diet do not show any indication that ingestion of sulfanilamide or sulfanilamide and alkali during salt deprivation causes alkalosis, although the urinary excretion of chloride and total base was essentially zero during sulfanilamide administration.

In the experiments in which animals were given sulfanilamide every four hours, there is further evidence to indicate that sulfanilamide does not cause severe disturbance in the acid-base equilibrium, although in these experiments the average blood level was considerably above that usually sought in clinical practice. In one of the animals (Dog 2) in which a marked dehydration developed as a result of vomiting, the serum chloride and serum total base concentrations fell by approximately 30 meq. per liter; however, the serum bicarbonate and serum pH were not markedly changed. In this experiment the collection of four-hour urine samples indicated that even though relatively high and constant blood sulfanilamide levels are maintained, the urine pH will still show considerable fluctuation. Marshall, Cutting, and Emerson[3] suggested that the excretion of an alkaline urine after sulfanilamide is due to the effect of sulfanilamide in preventing a reabsorption of bicarbonate in the kidney tubules. McChesny, Sprague, and Marshall[9] also believed this to be the case. On the other hand, Höber[12] has suggested that sulfanilamide causes an alkaline urine by reason of its inactivating effect on carbonic anhydrase. From the results that are summarized in Table IV, it would appear that other factors are also involved in regulating urine pH and that the secretion of an alkaline urine will not continue even though the blood sulfanilamide is maintained at a high and constant level. The acid-base equilibrium of the blood did not suggest any basis for the variation in urinary pH.

Marshall, Cutting, and Emerson[3] reported acid-base studies in three dogs that were given large doses of sulfanilamide. The findings suggested acidosis in some of the samples, although the results were not uniform. The dogs reported in Marshall's study showed marked signs of intoxication, as did those described in the present report which were given a single large dose of sulfanilamide. However, careful study of multiple blood samples in all three of the animals of the present study did not disclose any evidence of marked acidosis, although the serum bicarbonate was somewhat decreased. The marked dysfunction of the gastrointestinal tract and respiratory system that occurs following large doses of sulfanilamide gives a basis for disturbances in the acid-base equilibrium of the blood, although in the present experiments no marked disturbances were observed.

All of the present experiments offer evidence that the administration of sodium citrate along with sulfanilamide has no contraindications and may furthermore be desirable from the standpoint of preventing the decrease in serum bicarbonate that follows sulfanilamide ingestion.

SUMMARY

1. Studies of the acid-base balance of the blood were carried out on dogs during continued ingestion of sulfanilamide in amounts sufficient to maintain therapeutic blood concentrations. Small decreases in serum bicarbonate were observed, but there was no appreciable alteration in the serum pH.

2. Administration of sodium citrate along with the sulfanilamide prevented any decrease in serum bicarbonate and did not cause the pH to vary appreciably from the normal.

3. Ingestion of a low salt diet before and during a period when sulfanilamide or sulfanilamide and sodium citrate was ingested did not give results significantly different than were obtained during ingestion of a normal diet.

4. Maintenance of blood sulfanilamide concentrations at levels above those sought therapeutically did not produce any marked changes in the acid-base equilibrium of the blood.

5. Single massive doses of sulfanilamide, sufficient to produce signs of severe intoxication, did not cause marked changes in the acid-base equilibrium of the blood but did tend to cause a loss of water from the blood.

6. The pH of urine samples obtained at four-hour intervals during the maintenance of high blood sulfanilamide levels showed marked daily fluctuations which could not be correlated with certain views expressed in the literature.

REFERENCES

1. Free, A. H., Bowman, D. E., Davies, D. F., and Myers, V. C.: The Effect of Sulfanilamide on the Acid-Base Equilibrium of the Dog. J. Biol. Chem. 140: XLII, 1941.
2. Southworth, H.: Acidosis Associated With the Administration of Para-Amino-Benzene-Sulfonamide (Prontylin), Proc. Soc. Exper. Biol. & Med. 36: 58, 1937.
3. Marshall, E. K., Cutting, W. C., and Emerson, K.: The Toxicity of Sulfanilamide, J. A. M. A. 110: 252, 1938.
4. Strauss, M. B., and Southworth, H.: Urinary Changes Due to Sulfanilamide Administration, Bull. Johns Hopkins Hosp. 63: 41, 1938.

5. Hartmann, A. F., Perley, A. M., and Barnett, H. L.: A Study of Some of the Physiological Effects of Sulfanilamide. I. Changes in Acid-Base Balance, J. Clin. Investigation 17: 465, 1938.
6. Hartmann, A. F.: Clinical Studies in Acidosis and Alkalosis; Use and Abuse of Alkali in States of Bicarbonate Deficiency Due to Renal Acidosis and Sulfanilamide Alkalosis, Ann. Int. Med. 13: 940, 1939.
7. Beckman, W. W., Rossmeisl, E. C., Pettengill, R. B., and Bauer, W.: A Study of the Effects of Sulfanilamide on Acid-Base Metabolism, J. Clin. Investigation 19: 635, 1940.
8. Siebert, W. J., and Loose, F.: Comparative Studies on the Absorption of Sulfanilamide, J. Lab. & Clin. Med. 26: 371, 1940.
9. McChesney, E. W., Sprague, K. D., and Marshall, I. H.: The Effect of Sulfanilamide on Acid-Base Balance, J. Lab. & Clin. Med. 26: 1154, 1941.
10. Free, A. H., Davies, D. F., and Myers, V. C.: Effect of Sulfanilamide Accompanied by Acid or Alkali Upon the Acid-Base Equilibrium of the Dog. Possible Influence of Carbonic Anhydrase, J. Biol. Chem. 147: 167, 1943.
11. Nilson, H. W.: Corticoadrenal Insufficiency: Metabolism Studies on Potassium, Sodium and Chloride, Am. J. Physiol. 118: 620, 1937.
12. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. 115: 63, 1936.
13. Höber, R.: Effect of Some Sulfonamides on Renal Secretion, Proc. Soc. Exper. Biol. & Med. 49: 87, 1942.

PROTEIN HYDROLYSATE IN THE REGENERATION OF SERUM PROTEIN IN THE HYPOPROTEINEMIC RAT*

David B. Sabine, B.S., and Hans R. Schmidt, M.S., Yonkers, N. Y.

Recently there have appeared reports on the regeneration of various proteins in variously depleted animals. Some of these studies have had the aim of comparing high protein diets with equivalent amounts of protein hydrolysates[1] or of determining the relative efficiency of oral and intravenous administration.[1-3] The results of these investigations have shown, *first, that* hydrolysates are at least as efficient as whole protein; e.g., in the regeneration of plasma protein in dogs rendered hypoproteinemic by restricted diet,[1] and, second, that hydrolysates are as effective intravenously as orally.[1-3]

In the normal clinical treatment of hypoproteinemic patients (e.g., postoperative care, after more or less severe depletion) it is now not uncommon (for examples see ref. 4) to supplement a regular hospital diet with some protein hydrolysate, possibly an enzymatic digest administered orally (more rarely, and with attendant danger of shock,[3] parenterally) or an acid hydrolysate (fortified, perhaps, with tryptophane and cystine) injected intravenously. Accordingly, experiments were undertaken in this laboratory to determine whether or not a normal diet supplemented by an enzymatic protein hydrolysate would be superior to an unsupplemented diet in the regeneration of serum protein in hypoproteinemic albino rats.

*From the Biochemical Research Laboratories, The Arlington Chemical Company, Yonkers, N. Y.
Received for publication, January 15, 1943.

EXPERIMENTAL

Two sets of litter mates were used in the experiments. The first group, all males, weaned four weeks after the litter was cast, was kept on a diet of Purina Checkers until almost five months old; the second group, two males and four females (segregated as to sex after weaning), were similarly treated until almost four months old.

At this point both litters were put on *Diet 6A* (Table I). This diet, designed to deplete protein reserves, was fed *ad libitum*. Litter I was kept on this diet for 39 days; Litter II for 26 days. The animals at this time were distinctly hypoproteinemic and had lost much weight (cf. Table II). Each litter was then divided into two groups. One group received Regeneration Diet I (powdered Purina Checkers, total nitrogen 4.29 per cent), and the other, Regeneration Diet II (powdered Checkers (90 per cent) to which was added 5.5 per cent of a protein hydrolysate preparation* and 4.5 per cent cornstarch, total nitrogen 4.27 per cent). This latter mixture contained 10 per cent of its nitrogen in the form of the protein hydrolysate preparation.

On the protein regeneration diets serum protein levels were followed at approximately two-week intervals for Litter I during seven months, for Litter II during 4.5 months. The animals were weighed twice each week.

TABLE I

PROTEIN DEPLETION DIET 6A

Butter (salt)	8.95%
Lard	4.48%
Yeast	0.28%
Cod-liver Oil	2.24%
Osborne-Mendel Salt Mixture	4.48%
Cornstarch	67.25%
Sucrose	12.32%

TABLE II

WEIGHT AND SERUM PROTEIN VALUES

	LITTER I*					LITTER II†				
		GROUP A		GROUP B			GROUP A		GROUP B	
	AGE (DAYS)	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %	AGE (DAYS)	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %
Predepletion period	149	254	6.08	240	6.32	118	177	6.50	181	6.56
End of depletion period	188	166	4.99	162	4.90	144	113	5.43	117	5.35
Regeneration period	203	190	5.49	199	5.79	167	163	5.86	171	6.20
	217	203	5.61	230	5.87	182	177	5.82	187	6.24
	246	232	6.23	231	6.76	217	207	6.04	211	6.48
	318	252	6.14	268	6.33	273	209	6.42	221	7.16
	401	307	6.21	313	6.48	317	210	6.44	222	6.89

*Litter I—Group A, 2 males
Group B, 3 males

†Litter II, Each group, one male and two females
Groups A were on Regeneration Diet I
Groups B on Regeneration Diet II.

*The protein hydrolysate preparation used was Aminoids, a product containing 44.5 per cent of an enzymatic digest of dried powdered beef (15.6 per cent), wheat gluten (41.6 per cent), dried skim milk (41.6 per cent), and dried brewer's bottom yeast (1.2 per cent).

Serum protein was determined by the falling drop method, using a Kagan proteinometer,[5] on serum obtained from tail slices. All determinations were made in duplicate. In Table II are presented some of the data accumulated during these experiments. Full tabulations are omitted to conserve space.

The total consumption of Regeneration Diets I and II was measured, and the average food intake per rat was calculated from these figures. These data appear in Table III.

TABLE III

TOTAL AVERAGE DAILY FOOD CONSUMPTION PER RAT DURING REGENERATION PERIOD

	GROUP A	GROUP B
Litter I	14.35 Gm. (0.615 Gm. N)	14.42 Gm. (0.66 Gm. N)
Litter II	12.88 Gm. (0.552 Gm. N)	13.12 Gm. (0.560 Gm. N)

RESULTS AND DISCUSSION

In the case of Litter I where an average weight loss of 33.5 per cent and an average decrease of 20.4 per cent in blood serum protein concentration were recorded during the protein depletion period, the animals on Regeneration Diet I (without added hydrolysate) required a significantly longer time (123 days as opposed to 100 days) to recover the weight lost. With regard to the regeneration of serum protein, no attempt was made to estimate the amount of new serum protein formed, since blood volume studies were not undertaken. Using as an index the per cent of protein in the blood serum, it will be seen that this value rose at a significantly higher rate during the first 58 days, at least, for the animals on Regeneration Diet II (containing added hydrolysare).

The animals of Litter II showed slightly more severe weight loss (35.7 per cent) and slightly less decrease in per cent of protein in blood serum (17.5 per cent) at the end of the depletion period. Here, however, the weight recovery on both diets took place much more quickly (38 and 35 days on Regeneration Diets I and II, respectively). The only explanation which can be put forth from the data at hand seems to be that although the percentage decrease in blood serum protein content was almost as high as in Litter I, the actual value attained at the end of the depletion period was significantly higher, which suggests that these animals having been less hypoproteinemic were, consequently, in better general health. Also the animals in Litter II were about one month younger than those in Litter I and may still have been in the period of normal growth. Consideration of these factors makes even more striking the fact that the percentage of protein in the blood serum increased more rapidly on Regeneration Diet II than on Diet I.

Especially significant is the response recorded in the blood serum protein levels during the first intervals in each experiment. In Litter I the increases on Diets I and II were respectively 10 per cent and 18.1 per cent during the first 15 days; the corresponding values in Litter II were 7.9 per cent and 15.9 per cent in the first 23 days.

When one considers that a prompt response to diet therapy is the prime desideratum in the treatment of hypoproteinemia, the value of adding hy-

drolyzed protein to an otherwise normal diet is rendered impressive by this result. It has already been shown by other workers that hydrolyzed protein alone seems not to have any special advantage over unhydrolyzed protein as the dietary source of nitrogen in the regeneration of blood serum protein.[3] It is evident from these experiments that the combination of both is singularly effective and can be utilized effectively in what Davis and Getzoff[6] term posthepatic and prehepatic hypoproteinemia of certain types.

When these results are considered in the light of the data in Table III, it becomes noteworthy that the increases in blood serum protein levels were achieved in both litters without significant increases in the total intake of nitrogen; i.e., the effects observed were obtained by a substitution of hydrolyzed protein for intact protein, not by addition to an otherwise adequate diet.

SUMMARY

1. Rats rendered hypoproteinemic by diet restriction were observed to recover from the protein depleted state by oral administration of a normal diet, and of a similar diet in which hydrolyzed protein was substituted for 10 per cent of the intact protein.

2. With practically equivalent total consumption on both diets, regeneration, as measured by increase in the blood serum protein level, was practically twice as rapid on the fortified diet during the first two to three weeks of the regeneration period.

The authors are indebted to R. A. Harte for assistance in the preparation of this manuscript.

REFERENCES

1. Cox, Warren M., Jr., Mueller, Arthur J., and Kemmerer, K. S.: Serum Protein Regeneration as Effected by Intravenously Administered Protein Hydrolysates, Federation Proceedings 1: 106, 1942.
2. Robscheit-Robbins, F. S.: Amino Acids in Hemoglobin Formation, Federation Proceedings 1: 219, 1942.
3. Elman, R., Sachar, L. A., Horwitz, A., and Wolff, H.: Regeneration of Serum Albumin With Hydrolyzed Protein in Chronic Hypoproteinemia Produced by Diet, Arch. Surg. 44: 1064, 1942.
4. Beling, C. A., and Lee, R. E.: Treatment of Hypoproteinemia by Oral Administration of Protein Hydrolysate, Arch. Surg. 43: 735, 1941.
5. Kagan, B. M.: A Simple Method for the Estimation of Total Protein Content of Plasma and Serum, J. Clin. Investigation 17: 369, 1938.
6. Davis, H. A., and Getzoff, P. L.: Hypoproteinemia in Surgical Diseases, Arch. Surg. 44: 1071, 1942.

LABORATORY METHODS

GENERAL

IMPROVED METHOD OF TRANSPORTATION OF MATERIAL FROM GONOCOCCAL INFECTIONS*

Lenore R. Peizer, B.A., and Gustav L. Steffen, Ph.D., New York City

The use of gonococcus cultures has been considerably hindered by the lack of simple methods of transportation of gonococcal pus specimens to the laboratory. The method most used at present is to immerse a cotton swab containing the pus into broth or some other liquid medium in a carrying tube and to send it to the laboratory within six hours from the time the material was taken from the patient. When the culture material is in transit a longer time, negative culture results are unreliable.

Comparatively few investigators (Schofield,[1] Lumiere and Chevrotier,[2] Hauptman and Philadelphy,[3] Wortman, Gronau, Deakin, and Love,[4] Conn[5]) have studied the effect of time, temperature, and medium on the viability of gonococci in gonorrheal specimens. In the present study an attempt has been made to determine the influence of time and temperature on the survival of gonococci in specimens received on swabs immersed in broth, and to obtain data for the introduction of an improved transportation medium.

Material from known positive male and female cases of gonorrhea was used. The specimens reached the laboratory fifteen to thirty minutes after being taken. In the laboratory each swab was immediately streaked on plasma hemoglobin agar[6] and returned to the broth in the carrying tube. Two series of determinations were made; one series of 171 specimens in which the material was kept in broth at room temperature (22° C), and another of 162 specimens in which the specimens were kept at refrigerator temperature (6° C.). Following the inoculation of the immediate or control culture each specimen was plated on the same kind of medium every two hours for ten hours and again at twenty hours. The plates were incubated at 37° C. in an atmosphere of 8 to 10 per cent CO_2 for forty hours immediately after inoculation.

Table I shows the results obtained from examination of the two-hourly series of plates inoculated with known gonococcal material kept in broth at room temperatures (22° C.) and at refrigerator temperature (6° C.). The readings are separated into 4 plus (4+), 3 plus (3+), 2 plus (2+), 1 plus (1+), and plus-minus (±) according to the numbers of colonies found on the *first set* of streaked plates, which were the control plates of each series. In *Table I*, 3 plus and 1 plus readings were omitted since the results were similar to those

*From Bureau of Laboratories, Department of Health, New York City.
Received for publication, July 30, 1942.

TABLE I

NUMBER OF GONOCOCCUS COLONIES IN 66 FOUR PLUS SPECIMENS KEPT IN BROTH AT ROOM TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(4+) 200 COLONIES AND OVER	(3+) 100-200 COLONIES	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%						
After 2 hr.	97%	3%					
After 4 hr.	77%	19%	4%				
After 6 hr.	56%	21%	17%	6%			
After 8 hr.	40%	10%	30%	20%			
After 10 hr.	37%	13%	14%	20%	16%		
After 20 hr.	10%	0%	19%	25%	26%	10%	10%

NUMBER OF GONOCOCCUS COLONIES IN 36 TWO PLUS SPECIMENS KEPT IN BROTH AT ROOM TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%				
After 2 hr.	88%	12%			
After 4 hr.	36%	64%			
After 6 hr.	18%	75%	7%		
After 8 hr.	12%	44%	37%	0%	7%
After 10 hr.	12%	6%	62%	13%	7%
After 20 hr.	6%	8%	6%	64%	16%

NUMBER OF GONOCOCCUS COLONIES IN 27 PLUS-MINUS SPECIMENS KEPT IN BROTH AT ROOM TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%		
After 2 hr.	100%		
After 4 hr.	96%	4%	
After 6 hr.	54%	37%	9%
After 8 hr.	30%	50%	20%
After 10 hr.	0%	76%	24%
After 20 hr.			

NUMBER OF GONOCOCCUS COLONIES IN 50 FOUR PLUS SPECIMENS KEPT IN BROTH AT REFRIGERATOR TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(4+) 200 COLONIES AND OVER	(3+) 100-200 COLONIES	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%						
After 2 hr.	100%						
After 4 hr.	58%	25%	17%				
After 6 hr.	39%	20%	21%	20%			
After 8 hr.	33%	11%	27%	29%			
After 10 hr.	27%	8%	17%	33%	15%		
After 20 hr.	5%	6%	17%	14%	34%	24%	0%

NUMBER OF GONOCOCCUS COLONIES IN 59 TWO PLUS SPECIMENS KEPT IN BROTH AT REFRIGERATOR TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%				
After 2 hr.	100%				
After 4 hr.	60%	40%			
After 6 hr.	40%	53%	7%		
After 8 hr.	15%	59%	26%		
After 10 hr.	14%	36%	37%	13%	
After 20 hr.	7%	0%	46%	47%	0%

TABLE I—CONT'D

NUMBER OF GONOCOCCUS COLONIES IN 22 PLUS-MINUS SPECIMENS KEPT IN BROTH AT REFRIGERATOR TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE			
Incubated immediately	100%					
After 2 hr.	100%					
After 4 hr.	82%	18%				
After 6 hr.	73%	27%				
After 8 hr.	42%	58%				
After 10 hr.	12%	88%				
After 20 hr.	0%	100%	0%			

obtained from 4 plus and plus-minus cultures respectively. In considering the results of Table I it is essential to take into account the probable error in the percentages due to the mechanical factors involved in streaking a number of plates with one swab.

Out of a total of 171 positive specimens kept in broth at room temperature for twenty hours, 95, or 52 per cent, failed to show gonococci on culture. Of the 162 refrigerator specimens, 82, or 50 per cent, failed to show gonococci on culture.

The tests just described indicate that there is a diminution in the number of gonococci in storage of the specimen in broth, and that the diminution is in ratio to the length of time in storage. When specimens are kept in broth at room temperature, the culture frequently becomes overgrown with contaminating bacteria which prevent the isolation of the gonococci. Therefore when broth is used as a transportation medium, it is necessary to reduce the time in transportation to a maximum of four hours, particularly in doubtful cases.

In a subsequent study, in which plasma hemoglobin agar plates instead of broth were used for transportation, the following method was adhered to: A set of three plates was used for each known positive case. The plates were inoculated in the clinic and sent to the laboratory without delay. One plate from each specimen was incubated immediately at 37° C. for forty hours, in an atmosphere of 8 to 10 per cent CO_2. The second plate was kept at room temperature (22° C.) for twenty to twenty-four hours, and the third plate was placed in the refrigerator (6° C.) for twenty hours. At the end of twenty to twenty-four hours the room and refrigerator plates were incubated in the same manner as the immediate plate. The results obtained from the immediate and delayed cultures are shown in Fig. 1.

By transporting material on plasma hemoglobin agar, the loss of positives at room temperature after twenty to twenty-four hours was reduced from 52 per cent loss in broth to 11 per cent on agar, and at refrigerator temperature, from 50 per cent loss in broth to 14 per cent on agar.

Although transportation on solid medium is an improvement over the broth method, the loss of 11 to 14 per cent of positives in twenty to twenty-four hours is too great. Various substances were therefore incorporated in the medium in an attempt to find one which would effect better survival of the gonococci during transportation. When cysteine monohydrochloride, which had been used by

Cohn[5] in liquid media, was added to plasma hemoglobin agar to a concentration of 0.05 to 0.03 per cent, the gonococci in the specimen on these plates remained alive in practically every instance, when stored at room temperature for twenty to twenty-four hours before incubation.

In the preparation of the cysteine medium, a sterile 10 per cent aqueous solution of cysteine monohydrochloride is added to the melted proteose peptone agar after it has been cooled to 55° C., and before phosphate, plasma, and hemoglobin are added. This precaution is taken to avoid the formation of a precipitate. Cysteine monohydrochloride is added to a concentration of 0.03 per cent. Fig. 2

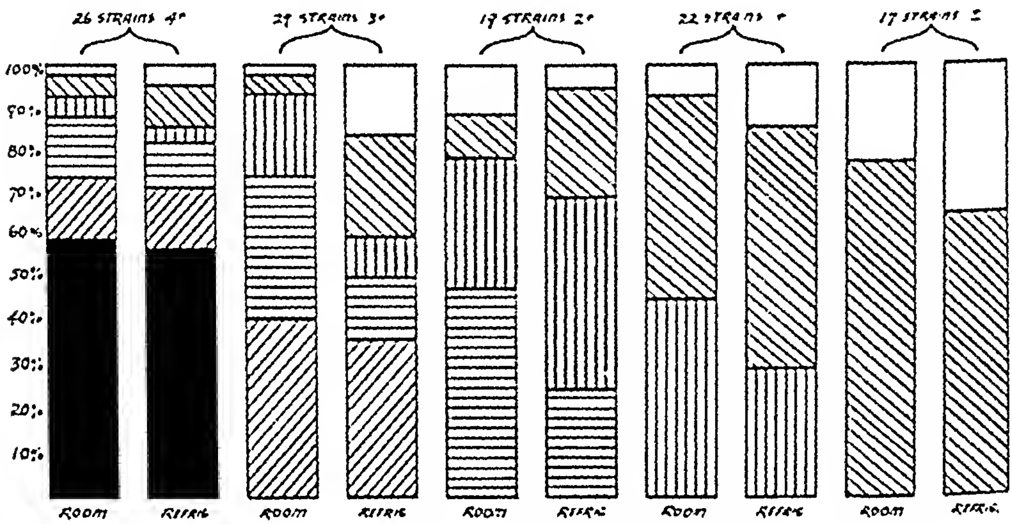


Fig. 1.—One hundred thirteen known positive strains from males streaked on horse plasma hemoglobin proteose peptone agar and left at room and refrigerator temperature for 24 hours before incubation.

Eleven strains lost at room temperature at end of 24 hours; 15 strains lost at refrigerator temperature at end of 24 hours.

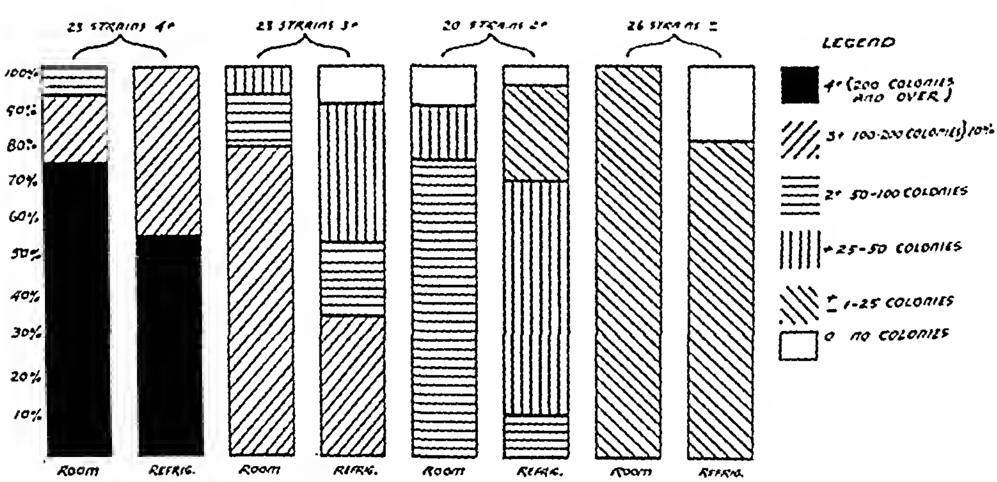


Fig. 2.—Ninety-two known positive strains from males streaked on 0.03 per cent cysteine monohydrochloride horse plasma hemoglobin agar and left at room and refrigerator temperature for 24 hours before incubation.

Eight strains lost at refrigerator temperature in 24 hours; none lost at room temperature.

shows the results on 92 known positive specimens from males, streaked on 0.03 per cent cysteine agar plates, and kept at room or refrigerator temperature for twenty to twenty-four hours. In this series no positives were lost at room temperature, and 8.7 per cent were lost at refrigerator temperature.

During this experiment l-cystine was also tested (Higginbotham[7]). This chemical inhibited the growth of gonococci when 0.1 per cent, 0.05 per cent, or 0.025 per cent was added to plasma hemoglobin agar. When 0.1 per cent was added to plasma hemoglobin agar containing veal or beef infusion as a base, it produced a luxuriant growth of gonococci. It seems that l-cystine requires a meat infusion base in order to stimulate good growth of gonococci. Because a meat infusion base is required and because the chemical dissolves only in the autoclave, l-cystine was not tested in transportation media.

The cysteine plasma hemoglobin agar was then tested as a transportation medium for general routine cultures. Each of 1,250 specimens were streaked directly on three plates, two of which contained plasma hemoglobin agar, and one, plasma hemoglobin agar with 0.03 per cent cysteine monohydrochloride. The plates without cysteine were incubated immediately as controls. The cysteine plate was kept at room temperature for twenty to twenty-four hours and then incubated in the usual manner. There were 240 positive cases, of which six control cultures were negative when the corresponding cysteine plates were positive, and five cysteine cultures negative when the corresponding control plates were positive. There was a drop in the number of colonies on cysteine plates in about 25 per cent of the positive cultures. This drop in number of colonies varies and is not in proportion to the number of colonies on the control plates. Almost all of the plus-minus cultures remained positive. The exposure of the streaked plates to out of doors winter temperatures for two to three hours did not affect the culture results.

SUMMARY

1. The streaking of gonococcal pus directly on to enriched solid medium containing 0.05 to 0.03 per cent of cysteine monohydrochloride saves 100 per cent of the positives when at room temperature up to ten hours, and about 98 per cent when left at room temperature from twenty to twenty-four hours.

2. In specimens kept in broth at 22° C. for six hours 10 per cent of positives are lost, and for ten hours 26 per cent of positives are lost. On plasma hemoglobin agar none are lost up to ten hours. When specimens are kept twenty hours at 22° C., 52 per cent of positives are lost in broth, 11 per cent on plasma hemoglobin agar, and only about 2 per cent on plasma hemoglobin agar with cysteine.

3. Transportation on solid medium is safer at 22° C. than at 6° C. Some cultures (8.7 per cent) are destroyed at refrigerator temperature when exposed to it longer than ten hours.

REFERENCES

1. Schofield, F. S.: Effects of Temperature on Viability of Gonococcus, J. Urol. 17: 581, 1927.
2. Lumiere, A., and Chevrotier, J.: Quelques aperçus nouveaux sur la bacteriologie du gonocoque, Bull. d. sc. pharmacol. 21: 385, 1914.

3. Hauptman, W., and Philadelphy, A.: Ueber das Kulturverfahren bei der weiblichen Gonorrhoe, Dermat. Wchnschr. 92: 495, 1931.
4. Wortman, M. S., Gronau, A., Beakin, R., and Love, F.: Preservation of the Gonococcus in Frozen Urines and Broth, Ven. Dis. Inform. 22: 195, 1941.
5. Cohn, A.: Experiments on Transportation of Gonococcal Pus for Culture Purposes, Am. J. Syph., Gonor., & Ven. Dis. 26: 598, 1942.
6. Peizer, L. R., and Steffen, G. L.: A Modification of the Horse Plasma Hemoglobin Agar for Primary Culture of the Gonococcus, Ven. Dis. Inform. 23: 224, 1942.
7. Higginbotham, M.: Culture Examination for N. Gonorrhoeae; Comparison of Chocolate Agar With Cystine Agar Medium, Am. J. Syph., Gonor., & Ven. Dis. 26: 607, 1942.

AN ELECTRICAL PROCEDURE FOR THE REPURIFICATION-CONCENTRATION OF ANTITOXIN*

Eugene Cardone, New York, N. Y.

When antitoxin ages, the antibody which it contains loses titer; this in all probability is a result of protein transformations which cannot be stopped. When a large decrease in antibody content occurs, the material becomes too weak for therapeutic use. Rather than discard this valuable material, it is desirable to increase its potency again by reducing its volume. The amount of total nitrogen possible per unit volume, however, is fixed by law, and therefore this reduction in bulk must be accompanied by a corresponding elimination of immunologically inert protein.

The salting-out method utilizing such salts as sodium and ammonium sulphate, first developed by Gibson,[1] and then improved upon by a host of others, is the most common method used today for purifying and increasing the titer of antitoxic plasma. Experience has shown that when this procedure is again employed in an attempt to repurify deteriorated antitoxin preparations, the losses sustained in active material are large and the increase in potency is small. In view of the fact that the englobulin, albumin, and fibrinogen fractions had been eliminated by the first purification of the antiserum, then, in the scope of this method, there is no more immunologically inert protein to be eliminated by repurification. Consequently an increase of antibody pseudoglobulin per unit volume is impossible.

An electrical process is described here which treats the antibody protein molecular aggregates in such a manner as to allow a further purification with a subsequent increase in antitoxin value per milligram of nitrogen. Furthermore, the color-carrying protein, which had been oxidized and reduced to colors varying from light brown to red, is to a great extent eliminated with the inert fraction, leaving the reprocessed material lighter in color.

The degree of increase in potency per unit of nitrogen has ranged with this process from 15 to 80 per cent. The variation is caused by the condition of the

*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York City.

Received for publication, August 3, 1942.

different lots reprocessed. Those which were old and whose protein content had become more stable did not allow as good a reconcentration as more recent material where protein transformations were more readily induced. The losses in antitoxin sustained by this process vary from less than 5 to more than 20 per cent depending again upon the condition of the material and upon the degree of reconcentration which one tries to attain.

The data in Table I give the variations in losses and in potency increase with six different lots of diphtheria antitoxin.

TABLE I

PREPARATION	ORIGINAL POTENCY IN GUINEA PIG UNITS AT 20% TOTAL SOLIDS (30.4 MG. N/GM.)	FINAL POTENCY IN GUINEA PIG UNITS AT 20% TOTAL SOLIDS (30.4 MG. N/GM.)	% LOSS IN TOTAL UNITAGE	% POTENCY INCREASE
1	3,540	4,580	6.6	29.3
2	3,540	5,420	4.1	53.1
3	1,190	1,840	0	54.6
4	1,630	1,970	0	20.8
5	1,120	1,740	28.7	55.3
6	1,137	1,940	0	70.6

The apparatus and procedure of the method are described here in detail because it has been found that even small variations in size of tubes, cellophane, amount of water added to the units, pH values, etc., make a decided difference in the quality of the final product.

APPARATUS NO. 4

The metal tubes are five inches long, one inch in diameter, and one thirty-second of an inch thick. They are made in the form of long, watertight thimbles. Zinc has been found to be the most efficient metal to use, because, for one reason, its ionization does not cause the serum to undergo rapid oxidation-reducing reactions accompanied by color formation.

The tubes are filled with tap water and the platinum electrodes are inserted as shown in Fig. 1.

There are two semipermeable membranes made of water-softened cellophane (Dupont, No. 600) and each contains three tube electrodes. Thus, there are three anode tubes in one membrane and three cathode tubes in the other.

The membranes are filled with tap water equal to the volume of antitoxin to be processed. The tube electrodes are immersed therein. For the support of these different units, the author has arranged a flat piece of glass with suitable holes to fit over the container, so that a metal frame firmly attached to each cellophane bag can be screwed onto it. Other holes allow the tube electrodes to be immersed in the fluid of each membrane.

The current used has been 120 volts direct current with the circuit as shown in Fig. 1. The current from a battery in conjunction with suitable resistance can be used instead, if desired. Three variable resistances are used instead of a large equivalent one for purposes of convenience only.

From 30 to 150 milliamperes are passed through the antiserum with an electromotive force of from 30 to 120 volts. This current, when used with this apparatus, has been found to cause no antibody destruction.

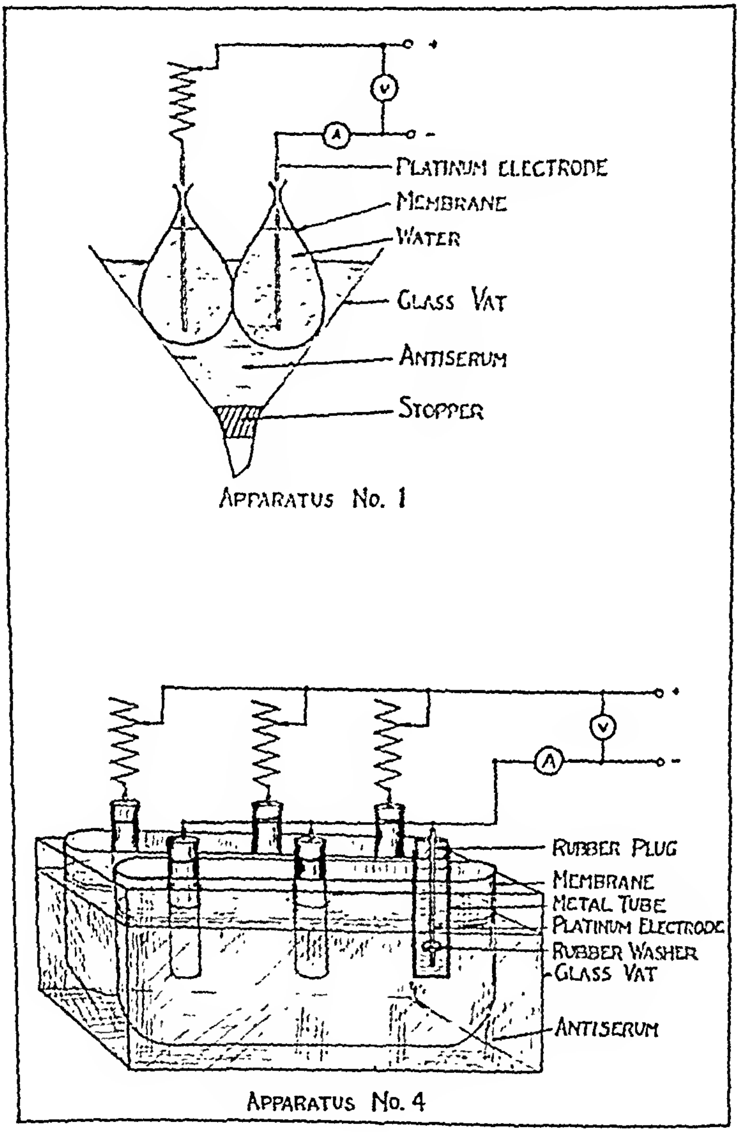


Fig. 1.

PROCEDURE

All hydrogen ion values referred to in the following description have been determined by the color comparator method. Gamma dinitrophenol and para and meta nitrophenol indicators with appropriate disc standards were used. This method has been checked with the potentiometric method and it has been found to be sufficiently accurate. It has been employed in this work because it lends itself more readily to the running of many determinations in a short time.

The antitoxin material to be processed is adjusted to a hydrogen ion value of pH 5.1 with normal acid acetic. It is dialyzed against running water until all the salts possible have been eliminated and the hydrogen ion value has increased to between pH 5.9 and pH 6.5. It is then diluted with distilled water to a nitrogen content of between 8 and 12 mg. of nitrogen per gram of material. The hydrogen ion value is again adjusted to pH 5.1 as above.

Thus prepared, the material is poured into the glass container of the apparatus, the membrane units are immersed therein, and the electric current is turned on.

When a preparation which permits visibility through it is being processed, one can immediately see stringlike oozing and settling of colloidal material all around and under the cathode membrane unit.

The material should be stirred from time to time manually unless a slow mechanical stirrer is used.

The hydrogen ion concentration must be checked often. It will be noted that as the process continues, more and more protein matter precipitates in the region of the cathode unit, accompanied by a slow but constant rise in pH value. When pH 5.9 has been attained in the mixture, the process is stopped.

It has been determined by many tests for antitoxin that the protein precipitated up to this point is mostly euglobulin. This fraction of protein is one of the immunologically inert globulin components which is precipitated between 0 and 30 per cent saturation with ammonium sulphate and whose existence has been further proved by the analysis of serum[3] with the Tiselius electrophoretic apparatus[4, 5] and the ultracentrifugal sedimentation method of Svedberg.[6] It contains no antitoxin except for a little which has been adsorbed by the precipitating proteins. This precipitate is separated from the mother liquor by filtration or centrifuging, and the precipitate is washed free of the adsorbed antitoxin by mixing it well with distilled water. The solid is sedimented or filtered again and is discarded. The washings are pooled with the filtrate or supernatant of the first separation.

In this manner the antitoxin has been repurified of an immunologically inert fraction of protein which, for the most part, would not have been present in the scope of the salting-out procedure.

The immunologically useful protein, namely, the pseudoglobulin, which remains in the clear purified material, can now be brought to a desired smaller volume by employing the following technique:

Normal sodium hydroxide is added slowly and with constant stirring to the purified colloidal solution. As the hydrogen ion concentration is reduced, more and more of the protein content precipitates out as in isoelectric precipitation. Finally at pH 7.0 to pH 7.4 all of the useful protein should be in the solid state. The precipitate is collected by filtration and the filtrate is discarded.

Normal acid acetic* is added slowly to the solid, with constant mixing, until the precipitate is in the colloidal state. The pH value of the resulting solution will be between pH 5.6 and pH 5.8. Solid sodium chloride to 1 per cent is added and the material is dialyzed against running water until only a trace of chloride

*An alkali has the same effect; however it has been found that antitoxin antibody is more tolerant to acidity than to alkalinity.

ion remains. One per cent solid sodium chloride and a suitable preservative are added after dialysis. The hydrogen ion value is set at pH 7.2 and the material is now ready for sterilization and standardization.

DISCUSSION

If according to Kruyt,[7] colloidal particles of the emulsoid type possess two factors which tend to make them stable with respect to precipitation, namely charge and hydration, and that either of these is enough to prevent aggregation of particles and flocculation of the colloid, then the apparatus as used satisfies both of these conditions and causes selective precipitation of the protein content of immune serum.

To develop the present equipment Apparatus 1 was first employed. Material processed with it showed oily, colloidal oozing and settling around and under the anode or cathode units depending on the direction of electrophoresis caused by the pH value of the antiserum.

When the process is begun with this apparatus at a neutral pH value, the settling of material at the anode unit is followed by the formation of a zone of clear fluid at the top of the serum in this region. The pH value of this clear fluid is acid and presently a hazy, ill-defined precipitate of pseudoglobulin separates out and slowly settles to the bottom layer. The clear zone then loses definition and assumes the pH value and the color of the bulk. There is no definite lasting precipitate, however, and the reaction of the serum during the process slowly becomes acid. This apparatus, therefore, although it causes protein transformations, is not enough of itself to cause aggregation of molecular particles and flocculation.

To add the necessary factor the metal jackets were added to the units, and their slow ionization completes the mechanism of the desired reaction. When antiserum at a neutral pH value is processed with this apparatus, the proteins precipitate in a thick coat on the outside surface of the anode membrane unit. When the process is begun with the serum at pH 5.1, however, the desired selective precipitation is obtained.

The hydrogen ion value of the fluids in the anode and cathode membrane units at the end of the process show the former to be between pH 6.2 and pH 6.5 while that of the latter is above pH 8.4. It has been found that an alkaline compound of the metal is formed in these units. The alkalinity produced in the anode unit, however, is transmitted to the serum as fast as it is formed by electrophoretic action. The result is a gradual rise in the pH value of the serum while that of the unit fluid itself remains constant. The cathode unit does not transmit any ion to the serum and therefore the reaction of this fluid becomes alkaline.

To demonstrate these points an alkali was added to the fluid of the anode unit, with or without metal jackets, and the pH value of the serum rose immediately. The reverse was true when an acid was added. When either acid or alkali were added to the fluid of the cathode unit no change in the pH value of the serum occurred.

That the anode metal jacket is the real functional one was shown by processing some material with a platinum electrode in the cathode unit and the

regular metal jacket in the anode unit. The serum underwent selective precipitation as usual, but the degree of reconcentration was not as good as when the complete apparatus was used. No selective precipitation occurred when the metal jacket was put in the cathode unit and the platinum electrode in the anode.

The anode tube is gradually used up during the process by the slow ionization of the metal while the outside surface of the cathode tube shows a black region, which is caused by a deposition of metal ion from the system as in electroplating.

The apparatus also causes some electrodialysis of the serum to occur making possible protein precipitation with very small quantities of metal ion.

SUMMARY

An electrical process is described which makes possible the repurification of deteriorated antitoxin material.

The apparatus is planned in a manner that allows purification and final reduction of volume by isoelectric precipitation of the proteins, thus eliminating the use of saturated salts.

The procedure is simple and it can be adapted to large-scale work.

REFERENCES

1. Gibson, R. B.: The Concentration of Antitoxin for Therapeutic Use, J. Biol. Chem. 1: 161, 1906.
2. Kruyt, H. R.: Colloids, ed. 2, New York, 1930, John Wiley and Sons, Inc.
3. Tiselius, A.: Electrophoresis of Serum Globulin: Electrophoretic Analysis of Normal and Immune Sera, Biochem. J. 31: 1464, 1937.
4. Tiselius, A.: The Moving Boundary Method of Studying the Electrophoresis of Proteins, Nova Acta Reg., Soc. Scient., Upsala. 7: 1930.
5. Tiselius, A.: A New Apparatus for Electrophoretic Analysis of Colloidal Mixtures, Faraday Society, Transactions 33: 524, 1937.
6. Svedberg, T.: The Ultracentrifuge and Its Field of Research, Indust. and Engin. Chem. (Analytical Edition) 10: 113, 1938.

   Svedberg, T.: Zentrifugierung, Diffusion und [illegible] loiden und hochmolekularen Stoffen, Kollo[illegible] 36: 53, 1925.

   Svedberg, T.: The Determination of Molecular Weight by Centrifuging, Z. physik. Chem. 65: 121, 1926.

   Svedberg, T.: The Determination of Molecular Weight by Centrifuging, Z. physik. Chem. 51: 127, 1927.

A STUDY OF MEDIA FOR TYPHOID BLOOD CULTURES*

THOMAS C. GRUBB, PH.D., BALTIMORE, MD.

INTRODUCTION

THE isolation and identification of *Eberthella typhosa* from the blood, feces, or urine of a patient has long been recognized as the most valuable laboratory aid in the diagnosis of typhoid fever. While extensive experimentation has yielded a number of very productive types of media for the isolation of *E. typhosa* from the feces and urine, relatively little choice of media is available for the isolation of this organism from the patient's blood. The glycerol-bile-peptone medium devised by Conradi[1] in 1906 is probably the most frequently used medium for typhoid blood cultures, particularly clot cultures, at the present time. It is commonly believed, as stated by Coleman,[2] that the bile in this medium dissolves the blood clot, but in the writer's experience little if any dissolution of the clot occurs during the usual incubation period of seven to ten days at 37° C. The popular belief in this property of bile is probably based upon the misinterpretation of Conradi's original statement that the bile *prevents the formation* of a clot when whole blood is inoculated into the medium.

The routine use of clot cultures for the diagnosis of typhoid fever has become a common practice in public health laboratories during recent years, since both an agglutination test and a blood culture can be performed on the same specimen. While this practice is usually satisfactory, there is apparently an unavoidable tendency for many of the clots to become contaminated either in the taking of the blood or during transportation to the laboratory. The writer is aware of public health laboratories where as high as 50 per cent of the clots have been contaminated, especially during the warm months, despite meticulous aseptic technique after the specimens reached the laboratory. Since the majority of contaminating organisms from clot cultures in Conradi's medium are staphylococci, it would appear desirable to add some substance to the medium which would suppress the growth of staphylococci without inhibiting the multiplication of the typhoid bacillus, if present. Recently Hager and Grubb[3] reported that p-bromocinnamic acid would exhibit such a selective inhibitory action against staphylococci without affecting the growth of *E. typhosa*. Since this preliminary study suggested the utility of p-bromocinnamic acid for typhoid clot culture media, an extensive investigation was made to determine the practicability of adding this acid to Conradi's medium. The present report summarizes the results of this study. In addition, a comparative study was made with media containing sodium desoxycholate to determine whether or not this agent, suggested by Sellers[4] for blood cultures, was more or less effective than p-bromocinnamic acid in selectively inhibiting the growth of staphylococci.

*From the Department of Bacteriology, University of Maryland, School of Pharmacy.
The writer is indebted to Miss Shirley M. Glickman for assistance in this work.
Received for publication, August 5, 1942.

EXPERIMENTAL

Preliminary experiments indicated that the selective inhibitory action of p-bromocinnamic acid for staphylococci was dependent upon the concentration of the acid and the pH of Conradi's medium to which it had been added. It was found that the lower the pH of the medium, the less the amount of p-bromocinnamic acid required to suppress the growth of staphylococci. Likewise, if sufficient p-bromocinnamic acid were added at a low enough pH, a point was reached where the growth of the typhoid bacillus as well as that of the staphylococci was completely inhibited. It was therefore obvious that the proper balance of pH and concentration of p-bromocinnamic acid must be found which would inhibit the growth of staphylococci without affecting the growth of *E. typhosa.* After several trials it was found that if Conradi's medium was adjusted to pH 5.0 with p-bromocinnamic acid in a concentration of 1:5000, the medium would exhibit the desired selectivity. Table I indicates the results obtained with this combination containing 10 per cent of defibrinated rabbit's blood. Freshly isolated strains of *E. typhosa** and *Staphylococcus albus* were inoculated into the medium and plate counts in nutrient agar made immediately after inoculation and after twenty-four hours' incubation at 37° C.

It is evident from the data shown in Table I that there is a definite bactericidal action on both strains of staphylococci, while the multiplication of the typhoid bacillus was apparently unaffected. It is to be noted that an extremely large inoculum was used in the above experiment.

TABLE I

SELECTIVE BACTERICIDAL ACTION OF P-BROMOCINNAMIC ACID (1:5000) IN CONRADI'S MEDIUM (pH 5.0) CONTAINING 10 PER CENT BLOOD

ORGANISM	TOTAL PLATE COUNT PER C.C. OF MEDIUM	
	IMMEDIATELY AFTER INOCULATION	24 HOURS' INCUBATION AT 37° C.
E. typhosa No. 1	940,000	51,000,000
E. typhosa No. 2	400,000	34,000,000
S. albus No. 1	21,000	40
S. albus No. 2	100,000	23,000
Control Medium Without P.Bromocinnamic Acid		
E. typhosa No. 1	1,060,000	20,000,000
E. typhosa No. 2	920,000	22,000,000
S. albus No. 1	570,000	46,000,000
S. albus No. 2	860,000	64,000,000

In order to determine the effect of various concentrations of blood upon the inhibitory action of p-bromocinnamic acid, defibrinated rabbit's blood was added to Conradi's medium in amounts varying from 1 to 30 per cent. When the concentration of blood exceeded 15 per cent, there was a definite reduction in the inhibitory activity of p-bromocinnamic acid, which became more pronounced in the higher concentrations of blood. It is difficult to estimate how much blood or serum is liberated in the usual clot culture, but for the purposes of experi-

*Freshly isolated strains of *E. typhosa* from blood cultures were obtained through the courtesy of Dr. C. A. Perry, Director, Bureau of Bacteriology, Maryland State Department of Health.

mentation a concentration of 10 per cent has been selected as representing an average amount and has been employed throughout the experiments reported here.

While a concentration of 1:5000 p-bromocinnamic acid apparently had no inhibitory effect upon the growth of *E. typhosa* when a very large inoculum was used (400,000 to 940,000 per c.c. as shown in Table I), it was deemed advisable to determine the effect of this concentration upon a small inoculum. The number of organisms which should be used as an inoculum to simulate the number found in the blood stream of a typhoid patient is probably very small. Müller and Gröff[5] made plate counts from blood cultures of 256 typhoid cases and found that 82.2 per cent of the cultures contained between 1 and 50 organisms per c.c. of blood. Plate counts made on the blood of 43 patients in the University of Maryland Hospital showed between 1 and 63 typhoid bacilli per c.c. of blood; however, when a number of hours intervenes between the taking of the blood and the laboratory examination, such as occurs when specimens are sent through the mail to public health laboratories, it is difficult to predict what degree of multiplication will take place. For experimental purposes, inocula containing from 10 to 20 organisms were employed, and this small number of typhoid bacilli was markedly inhibited in Conradi's medium adjusted to pH 5.2 and containing 1:5000 p-bromocinnamic acid.

Since Sellers[1] had reported the use of desoxycholate broth for the culture of clots from suspected cases of typhoid fever, it was considered of interest to determine how this medium compared with the p-bromocinnamic acid medium described above. The formula for this medium, kindly supplied by Harris,[6] is as follows: peptone, 1 per cent; Na_2HPO_4, 0.35 per cent; sodium desoxycholate, 0.1 per cent. The hydrogen-ion concentration of the medium is adjusted to pH 7.0 to 7.2 before autoclaving at 15 pounds' pressure for fifteen minutes. Small inocula (10 to 25 organisms) of two recently isolated strains of staphylococci and *E. typhosa* were inoculated into the p-bromocinnamic acid and the desoxycholate media, and incubated 24 hours at 37° C. when plate counts in nutrient agar were made. The results of this experiment, which was repeated several times, are given in Table II.

It is evident from the data in Table II that while p-bromocinnamic acid is bactericidal for staphylococci, it is also markedly inhibitory for typhoid bacilli when a small inoculum is employed. On the other hand, the desoxycholate broth completely suppressed the growth of staphylococci while allowing considerable growth of *E. typhosa*. These data indicate clearly that sodium desoxycholate is a much more effective substance than p-bromocinnamic acid for the selective inhibitory action on staphylococci in typhoid blood cultures.

To determine what effect variations in the pH of the medium and concentration of sodium desoxycholate would have upon the selective inhibitory action of the desoxycholate broth, various portions were prepared containing from 0.05 per cent to 0.3 per cent sodium desoxycholate with the hydrogen-ion concentration of the broth ranging from pH 6.0 to 8.0. These portions, containing 10 per cent defibrinated rabbit's blood, were inoculated with approximately 10 staphylococci or typhoid bacilli per 5 c.c. of medium and incubated

twenty-four hours at 37° C. Each portion was then plated in nutrient agar to determine the total number of organisms present. The results of this experiment are shown in Table III.

TABLE II

COMPARISON OF THE BACTERICIDAL ACTION OF P-BROMOCINNAMIC ACID IN CONRADI'S MEDIUM WITH THAT OF DESOXYCHOLATE BROTH

MEDIUM	TOTAL PLATE COUNT PER C.C. OF MEDIUM AFTER 24 HOURS' INCUBATION AT 37° C.			
	ORGANISM		ORGANISM	
p-bromocinnamic acid (1:5000)	*S. albus* No. 1	0	*E. typhosa* No. 1	73
	S. albus No. 2	0	*E. typhosa* No. 2	1,070
Sodium desoxycholate (1:1000)	*S. albus* No. 1	0	*E. typhosa* No. 1	500,000
	S. albus No. 2	0	*E. typhosa* No. 2	700,000

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM DESOXYCHOLATE AND PH OF THE MEDIUM ON THE GROWTH OF *E. Typhosa* AND *S. Albus* IN DESOXYCHOLATE BROTH

ORGANISM	pH	TOTAL PLATE COUNT PER C.C. OF MEDIUM AFTER 24 HOURS' INCUBATION AT 37° C.			
		0.05%*	0.1%	0.2%	0.3%
S. albus No. 1	6.0	0	0	0	0
	7.0	0	0	0	0
	8.0	0	0	0	0
E. typhosa No. 1	6.0	22,100,000	48,000,000	25,000,000	28,000,000
	7.0	30,000,000	69,000,000	35,000,000	71,000,000
	8.0	11,000,000	24,400,000	71,000,000	34,000,000

*Concentration of sodium desoxycholate.

The data in Table III show the complete inhibition of growth of staphylococci while *E. typhosa* grew luxuriantly. Nutrient broth control tubes (pH 7.0) containing 10 per cent blood inoculated at the same time and incubated in a similar manner contained 153,000,000 staphylococci and 130,000,000 typhoid bacilli per c.c. of the culture. Thus while the sodium desoxycholate in the concentrations employed in this experiment has some inhibitory action on *E. typhosa*, it is probably insignificant so far as the practical usefulness of the medium is concerned. While the number of typhoid bacilli developing in the various concentrations of sodium desoxycholate at different pH values varied from 11,000,000 to 71,000,000 per c.c., there is no apparent relationship between the two variables and the number of organisms developing. It is believed that for practical purposes the desoxycholate broth will exhibit adequate selective inhibitory action in any of the combinations of pH and in the concentration of sodium desoxycholate shown in Table III. The fact that neither the concentration of the sodium desoxycholate nor the pH of the medium requires critical adjustment increases the practical usefulness of the medium.

Since it was found that the inhibitory action of p-bromocinnamic acid on staphylococci was nullified to a considerable extent by concentrations of blood exceeding 15 per cent, it was considered of practical importance to determine whether or not blood had a similar effect in the desoxycholate medium. Defibrinated rabbit's blood in concentrations ranging from 1 to 30 per cent was added

to desoxycholate broth containing 0.1 per cent sodium desoxycholate and adjusted to pH 7.0. Small inocula (100 to 200 organisms) of *E. typhosa* and *S. albus* were inoculated into the broth containing various amounts of blood and incubated at 37° C. for twenty-four hours when plate counts in nutrient agar were made. Even in the media containing 30 per cent blood there was no reduction in the inhibitory action of sodium desoxycholate on staphylococci, while in the media containing only 1 per cent blood, there was no inhibitory action on the growth of typhoid bacilli. This experiment indicates that variations in the concentration of blood, within the probable limits found under practical conditions, do not affect the selective inhibitory action of sodium desoxycholate in the medium described.

While sodium desoxycholate was shown to display an effective bactericidal action on staphylococci in previously described experiments where small inocula were used, it appeared advisable to determine the inhibitory action of this substance against a large inoculum, since considerable multiplication of contaminating staphylococci may occur when the blood specimen is sent through the mail to the diagnostic laboratory. In order to study this problem, 0.1 per cent desoxycholate broth adjusted to pH 7.0 and containing 10 per cent blood was prepared. Varying numbers of staphylococci were inoculated into tubes containing 5 c.c. of the medium and incubated twenty-four hours at 37° C. when plate counts were made to determine the number of surviving organisms. The results of this experiment are shown in Table IV.

TABLE IV

ACTION OF DESOXYCHOLATE BROTH UPON VARIOUS NUMBERS OF *Staphylococcus albus*

NUMBER OF ORGANISMS INOCULATED INTO 5 C.C. OF MEDIA	NUMBER OF ORGANISMS VIABLE AFTER 24 HOURS' INCUBATION AT 37° C.
12,800,000	4,000
1,280,000	3,050
128,000	450
12,800	50
1,280	0
128	0

This experiment demonstrated that sodium desoxycholate has a very effective bactericidal action against large numbers of staphylococci; and even though some of these organisms do survive over a 24-hour period, the number of surviving organisms is insignificant compared to the number of typhoid bacilli which grow during this period under the same conditions (see Table III).

DISCUSSION

The experiments described in this report indicate that while p-bromocinnamic acid shows a selective inhibitory action against staphylococci when the concentration of the acid and the pH of the medium are properly adjusted, this selective activity probably cannot be utilized in a medium for the culture of blood clots because small numbers of typhoid bacilli are inhibited. On the other hand, a simple broth containing from 0.05 to 0.3 per cent sodium desoxycholate and adjusted to pH 6.0 to 8.0, shows a remarkably clear-cut bactericidal

action against staphylococci without a significant degree of inhibition for the typhoid bacillus. This selective activity is manifest despite wide variations in the amount of blood present and despite an enormous inoculum of staphylococci. These experiments suggest that a desoxycholate broth, similar to that described, should prove very helpful to laboratories where difficulty is experienced in avoiding or eliminating contamination of blood clot cultures with staphylococci.

SUMMARY

1. A study was made of the utility of p-bromocinnamic acid incorporated in Conradi's medium for the purpose of inhibiting the growth of contaminating staphylococci. Although p-bromocinnamic acid showed a selective inhibitory action against staphylococci, it also inhibited small numbers of typhoid bacilli and therefore was not considered of practical value for the culture of blood clots from suspected cases of typhoid fever.

2. A simple broth containing sodium desoxycholate displayed an effective inhibitory action against staphylococci without an appreciable bacteriostatic effect upon the typhoid bacillus. The broth does not require critical adjustment of pH or concentration of sodium desoxycholate, and is effective in various concentrations of blood against large numbers of staphylococci.

REFERENCES

1. Conradi, H.: Ein Verfahren zum Nachweis der Typhuserreger im Blut, Deutsche med. Wchnschr. 32: 58, 1906.
2. Coleman, N. B.: Eberthella Typhosa and Other Bacillary Incitants of Enteric Disease, Diagnostic Procedures and Reagents, Amc. Pub. Health Assoc. ed. 1, 239, 1941.
3. Hager, G. P., and Grubb, T. C.: A Study of the Bacteriostatic Activity of Fluoro and Bromo Derivatives of Some Organic Acids, J. Infect. Dis. 71: 228, 1942.
4. Sellers, T. F.: Practical Procedures in the Laboratory Diagnosis of Typhoid and Clinically Related Fevers, Amc. J. Pub. Health 27: 659, 1937.
5. Müller, R., and Gröff, H.: Wert der Blutuntersuchung für die Typhusdiagnose, Centralbl. f. Bakt. (Abt. Orig.) 43: 856, 1907.
6. Harris, M. M.: Personal communication.

MICROSCOPIC OBSERVATION OF COLLODION PARTICLES AS INDICATORS OF TYPE-SPECIFIC PNEUMOCOCCIC IMMUNE REACTIONS

MARTIN BURGER, M.SC.
NEW YORK, N. Y.

THE object of this study was to detect the presence of minute amounts of pneumococcal soluble-specific substances (S.S.S.) in vitro. A sensitive microscopic technique was developed which was compared with a slightly modified method of Goodner's[1] and with the ordinary precipitation tests.

In 1932 Freund[2] demonstrated that anthrax soluble carbohydrate hapten was adsorbed by colladion particles; for on adding anthrax-immune serum to the hapten-sensitized particles, agglutination resulted. The agglutination was observed best in a hanging-drop preparation, but the titers of the reagents were not reported.

Experimental.—Collodion particles were prepared according to the method of Cannon and Marshall[3] except that double-distilled rather than triple-distilled water was used, as recommended by Eisler.[4] The particles obtained from 75 ml. of 5 per cent acetone stock solution, after aspirating, washing, and centrifuging, were suspended in 100 ml. of double-distilled water and used without further diluting.

Into agglutination tubes was added 0.02 ml. of collodion suspension followed by 0.1 ml. of S.S.S. dilution. The latter was made up in physiologic saline. After thorough mixing, the tubes were allowed to stand for from five to ten minutes at room temperature, then 0.1 ml. of antiserum added. The contents were mixed again, capped with rubber stoppers, and placed in the refrigerator overnight. With each test, a serum control (0.02 ml. collodion suspension plus 0.1 ml. antiserum) and an S.S.S. control (0.02 ml. collodion suspension plus 0.1 ml. S.S.S. dilution) were made. Another set of tubes containing the same amounts of reagents were simultaneously made up for the so-called "collodion-fixation test" of Goodner.[1] A third set without collodion particles constituted the ordinary precipitation test.

Microscopic Technique.—A drop of the collodion plus antigen (S.S.S.) plus antiserum suspension is placed on a plain glass slide. Next to it is placed a drop of the serum plus collodion control and next to this, a drop of the antigen plus collodion control. Each drop is covered with a coverglass and examined under the oil immersion lens. It is desirable to flip each tube gently about ten times just before withdrawing the drop of suspension. In the mounts containing the higher concentrations of S.S.S., one sees definite amorphous masses containing enmeshed collodion particles. These are designated 4 plus and 3 plus in Table I. A 2 plus reading signifies the presence of relatively large clumps

From William Hallock Park Laboratories, New York City Department of Health, Bureau of Laboratories, New York, N. Y.
Received for publication, Sept. 2, 1942.

of collodion particles without a trace of the amorphous precipitate. A plus reading means that the majority of the fields examined contain more clumps (small in size) and fewer single particles as compared with the controls. With one exception, all the serum and antigen controls thus far examined contained some small collodion clumps and many single particles; that is, the dispersion was good. The exception, a concentrated horse serum plus collodion control, contained large clumps of particles and was similar to a 2 plus reading.

The results of a typical comparative test are shown in Table I.

TABLE I

TITRATION OF S.S.S. III AGAINST A RABBIT TYPE III ANTIPNEUMOCOCCUS SERUM (UNDILUTED): COMPARATIVE TESTS

TUBE	DILUTION OF S.S.S.III 1:	MICROSCOPIC READING	MACROSCOPIC READING	PRECIPITATIVE TEST WITHOUT COLLODION
1	5×10^{-5}	4+	3+*	2+
2	10^{-6}	4+	2+†	+
3	3×10^{-6}	4+	+	±
4	5×10^{-6}	3+	±	–
5	5×10^{-7}	2+	–	–
6	10^{-9}	–	–	–
Serum control		–	–	–
S.S.S. controls	5×10^{-5}	–	–	–
	5×10^{-6}	–	–	–

*Disc.
†Coarse particles, not easily broken up on vigorous flipping.

SUMMARY

A microscopic method of observing collodion particle agglutination is described. In higher dilutions of pneumococcus S.S.S., the method gives positive results, while negative results are obtained by macroscopic examination either of the same tests or of ordinary precipitation tests.

REFERENCES

1. Goodner, K.: Collodion Fixation: A New Immunological Reaction, Science 94: 241-242, 1941.
2. Freund, J.: A Method of Immunization With Carbohydrate Haptenes Adsorbed on Collodion Particles, Science 75: 418, 1932.
3. Cannon, P. R., and Marshall, C. E.: An Improved Serologic Method for the Determination of the Precipitative Titers of Antisera, J. Immunol. 38: 365-376, 1940.
4. Eisler, D. M.: Influence of Collodion Particles on the Visible End-Point in Antibody Titrations, J. Immunol. 42: 405-418, 1941.

CHEMICAL

A METHOD AND APPARATUS FOR SHELL FREEZING AND RAPID DRYING OF PLASMA AND OTHER PRODUCTS FROM THE FROZEN STATE BY LOW TEMPERATURE WATER VAPOR CONDENSATION IN VACUO*

MAX M. STRUMIA, M.D., AND JOHN J. MCGRAW, M.D., BRYN MAWR, PA.

INTRODUCTION

HUMAN plasma transfusion is now generally accepted as an important therapeutic agent in the treatment of shock, associated with trauma, hemorrhage, or burns as well as of hypoproteinemias, hypoprothrombinemias, and of some hemorrhagic conditions. Preservation of plasma in the frozen state has been recommended as the safest, most efficient, and practical method for the average hospital.[1] The establishment of "plasma banks" in which the plasma is maintained frozen will allow most institutions to meet the routine or emergency needs of the communities which they serve in almost all instances. This is true of the present national emergency. "Banks" of frozen plasma are being established in many hospitals throughout the country in view of the increasing danger of industrial accidents associated with large scale production and the possibility of enemy action within the United States. Large stores of dried plasma are being prepared for use with the Armed Forces beyond the limits of the continental United States. Conditions do exist, and further situations may arise, in which a certain amount of dried plasma is needed for civilian use.

Very few hospitals at present are in a position to offer the use of dried plasma for their patients, except through outright purchase of the material. This situation in effect denies the patient the opportunity of availing himself of the use of friends and relatives as volunteer donors. In a previous publication[1b] it has been pointed out that this difficulty could be overcome if a group of hospitals would cooperate in the preparation of dried plasma. Since the preservation of plasma in the dried state is only seldom essential in the ordinary civilian practice, a single large institution could easily dry a sufficient quantity to meet the need of several others on a cooperative nonprofit basis. Almost any hospital can easily maintain its own store of frozen plasma, which would be supplemented by a small amount of the dried material supplied by the drying center. Under favorable conditions, a large institution may assume the responsibility for supplying plasma in both frozen and dried states for neighboring institutions.

*From the Bryn Mawr Hospital, Bryn Mawr, Pa.

Received for publication, January 11, 1943.

A plan of this nature has been operating successfully for over eighteen months in the Cincinnati General Hospital under Dr. Hoxworth.[16] The service supplies the needs of fourteen hospitals with a total bed capacity of 4,235. A state-wide plasma service is being organized by Dr. James of the University of Vermont Medical College.[17]

The purpose of this paper is to describe a method and an apparatus for the drying of plasma from the frozen state by low temperature water vapor condensation in vacuo suited for the plan just mentioned.

While the essential details of construction are given, no attempt is made to describe the apparatus minutely. Likewise, no attempt is made to give details of performance under all conditions of temperature, vacuum, etc., nor to justify many statements made concerning newer methods employed, such as the shell freezing at relatively higher temperatures, etc.

These phases of the work will be covered in subsequent papers.

A DISCUSSION OF PREVIOUS APPARATUS AND BASIC PRINCIPLES INVOLVED

Up to the present, all of the various apparatus described for the desiccation of plasma from the frozen state have been either too small for practical use or too expensive if large enough to serve the purpose. Some apparatus, although inexpensive to purchase, have proved a financial burden because of the operating expense and the need of much technical supervision. Most of these apparatus have been recently critically reviewed by Harkins.[2]

Of all the methods in use, we feel that desiccation from the frozen state in the final container is preferred, because this method provides for maximal preservation of all elements of the plasma, maximum solubility, minimal opportunity for contamination during the process of drying, and regeneration with 0.1 per cent citric acid prior to use.

It is important to note that the pH of plasma dried from the frozen state and restored with distilled water varies from 8.2 to 9.3. The same material regenerated with 0.1 per cent citric acid will have a pH varying from 7.4 to 7.8.[15]

The use of low temperatures for the condensation of the water vapor removed from the frozen plasma is preferred because it is efficient and relatively inexpensive. In the apparatus to be described here, the same source of low temperature is used for both prefreezing of the plasma in shell form and for water vapor condensation during the process of drying. The source of low temperatures is obtained by expansion of compressed Freon-12 gas in evaporator coils or plates. It is based on the fundamental principles employed by the early workers in this field.[3] In addition, the successful operation of this apparatus involves five important facts, which previously had not been generally accepted or taken advantage of:

1. The temperature of freezing of plasma has little or no practical effect, within the experimental limits (minus 12 to minus 72° C.), on the quality of the final product.

2. The temperature at which water vapor condensation is carried out is not critical, within the experimental limits (minus 30° to minus 72° C.), provided that the rate of evaporation at any time during the operation is sufficiently

rapid to avoid thawing of the frozen plasma. This is readily accomplished by using a condenser possessing a condensing surface sufficiently large and by taking advantage of certain details of construction of the condensing chamber pointed out later.

3. The temperature of the condensing coil during the period of maximal sublimation may rise somewhat above the minimum mentioned (minus 30° C.) without adversely affecting the process, provided that during the last few hours of the process the temperature is maintained between minus 30° C. and minus 35° C. or below.

4. The temperature of the water jacket heating the plasma may be raised, during a portion of the process, to relatively high temperatures (80° C.) without impairment of any of the essential qualities of the resulting product, but with considerable shortening of the time of drying as compared with apparatus without proper thermic control.

5. The temperature of the plasma itself may be raised to relatively high temperatures (75° to 80° C.) without deterioration, provided that the amount of moisture present is relatively low. This occurs in the last few hours of the process.

The demonstration of these facts has allowed the development of an efficient, practical apparatus, which will fit the program of plasma drying mentioned above. The use of very low temperatures (about minus 70° C.), which has been emphasized for both prefreezing and drying, has been one of the main factors in the almost prohibitive cost of other apparatus. Several types of inexpensive standard refrigerating machines on the market are capable of producing constant temperatures in the nature of minus 40° C. and thus allow the construction of a satisfactory apparatus at a low cost.

In the construction of the apparatus, the following practical points were kept in mind:

1. The product obtained should, when regenerated with solvent, be as nearly as possible identical with the original material. The apparatus and process employed, therefore, must allow no degeneration to occur during the process of drying. The plasma obtained with the apparatus here described in all ways complies with the regulations set forth by the National Institute of Health.

2. The apparatus must be so designed and the operation so controlled as to be constant, thus insuring a uniform product. It must be remembered that drying from the frozen state may be achieved in a great many ways and with much simpler apparatus. However, mechanical controls of temperature, recording devices for temperature and pressure, and other refinements of construction play an important role in insuring a uniformly good product.

3. The apparatus must be simple and economical in its operation, as much as possible automatic, so as to need a minimum of supervision once under way. It should provide facilities for shell freezing as well as for drying. To achieve simplicity and economy of operation, the apparatus, paradoxically, has to be relatively expensive and complicated.

It has been pointed out that drying from the frozen state may be achieved with a simple and inexpensive apparatus.[4] Such an apparatus, however, has a

limited capacity, requires much technical supervision, and is high in operating cost. The apparatus to be described here was so designed as to have a capacity large enough for the need of several hospitals. It requires very little attention during the operation, and the operating expense is negligible.

Thus the standard apparatus here described freezes and dries 24 units of citrated plasma of 300 c.c. each daily (7.2 liters), or 8,760 yearly (2,628 liters). The total cost of electric energy for shell freezing and drying is well under $.02 per unit, or less than $175.00 yearly. For the same operation, if carbon dioxide ice is employed, the yearly cost of refrigeration would be about $3,006.00, or considerably more than the entire cost of purchase of the apparatus.*

In order to evaluate properly the construction and performance of this apparatus, it is necessary to review the general basic principles of drying biologic substances. The Bordas and d'Arsonval apparatus,[3b] as reconstructed by us, demonstrates these basic principles in a simple form (Fig. 1). It consists of two glass flasks connected by a T-tube. One flask (A) contains a thin layer of material to be dried. The other flask (B) is the condenser. The T-tube is connected to a vacuum pump by means of a rubber tube (C), which may be closed by a stout pinch clamp. The glass tube connecting the two flasks must be of sufficient size to allow free passage of water vapor from A to B. Flask A is immersed in water kept at plus 15° C., and Flask B, acting as condenser, is cooled by immersion in carbon dioxide ice-alcohol mixture, maintaining a temperature of about minus 70° C.

Containers A and B are stout-walled Erlenmeyer flasks of about 500 c.c. capacity. Under these conditions, when 30 c.c. of plasma are introduced in Flask A, "snap" freezing, from the heat loss due to rapid evaporation, occurs in about five minutes after starting of the vacuum pump; thereafter, drying proceeds by sublimation of water from the frozen state. The water vapor is condensed on the cold walls of Flask B as fast as it is formed; thus it is not allowed to mix with the oil of the pump, which would rapidly reduce the efficiency of the pump. The dried plasma thus obtained appears in the form of irregular scales, has a residual moisture of less than 1 per cent, and is readily soluble.

Practically all of the various types of apparatus in general use for drying under a vacuum are based upon variations in the method of condensing the water vapor.

In the original apparatus used by Bordas and d'Arsonval, liquid air or carbon dioxide snow-acetone was used as refrigerating agent.

Vansteenherghe[3a] used sulfuric acid to absorb the water vapor instead of low temperature; Reichel[5] and Flosdorf and Mudd[6] used an apparatus similar to that of Bordas and d'Arsonval and CO_2 ice as the refrigerating agent for the "lyophile" process; later Flosdorf and Mudd[7] returned to the use of a chemical dehydrating agent and employed calcium sulfate in their "cryochem process"; Hill and Pfeiffer[8] used silica gel in their "adtevac" process.

Elser[3f] was the first to report the use of coils refrigerated by the expansion of compressed Freon-12 gas for condensation of water vapor. In actual opera-

*The cost of CO_2 ice is computed at $.03 per pound, the lowest prevailing price when purchased in large quantities. The amount of dry ice required is based upon our experience with a similar apparatus.[12]

tion, minus 34° C. to minus 36° C. were the lowest temperatures attained. Elser pointed out that the loss in efficiency from the change of the condenser temperature from minus 70° C. to minus 32° C. was relatively slight, roughly 5 per cent.

The failure of the mechanical refrigeration system of Elser has generally been laid to the too small differential in temperature between the substance being dried and the condensing surface. This is not confirmed by our findings, since satisfactory drying with a residual moisture of less than 1 per cent has been obtained with a maximal differential temperature of only 65° C.

We have already pointed out that the temperature of prefreezing is not critical within the experimental limits (minus 12° C. to minus 72° C.). With the apparatus here described, prefreezing and drying are carried out at much higher, and therefore much more convenient and economical, temperatures than generally employed without essential loss of quality of the material being dried.

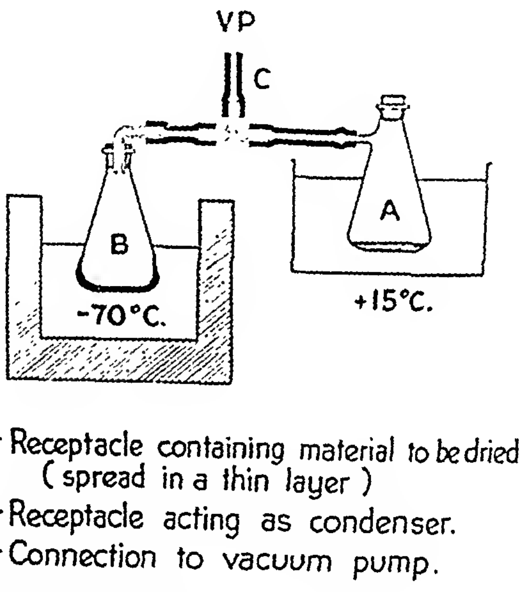


Fig. 1.—Bordas and d'Arsonval Apparatus for the drying of easily alterable substances.

Greaves and Adair[9] likewise found that the lowering of the condenser temperatures lowers the temperatures of the frozen material being dried to a very slight extent only. They successfully employed as refrigerating agent methyl chloride, and obtained temperatures of minus 52° C. to minus 42° C.

Greaves and Adair state that with temperatures of minus 45° C., the percentage of water remaining at the end of desiccation is much less than when higher temperatures are employed. We have found that with the water jacket of the drying chamber at plus 35° C. to plus 36° C. and temperatures of condensation of minus 30° C. to minus 35° C., the residual moisture of the resulting material is constantly less than 1 per cent.

The method employed for the determination of the residual moisture is that described by the National Institute of Health, i.e., drying of the material in high vacuum over phosphorus pentoxide to constant weight.

The great advantages in the employment of relatively high temperatures of water vapor condensation are, essentially, simplicity of operation and economy, since the heat neutralizing capacity of a compressor falls rapidly with the lowering of the temperature of condensation.

There are two methods of drying under vacuum which do not employ a condenser. One of these, the "desivac" machine,[10] allows the water vapor to enter the vacuum pump and constantly removes the water from the oil by means of a high speed centrifuge. The high cost of this apparatus and the relatively long drying time make its operation uneconomical. Another method produces a vacuum by means of multiple steam jets, in which case the water vapor escapes with the steam, thus eliminating the need for a condenser. This method is particularly suited to large quantity production on a commercial scale.[11]

With the possible exception of the method of Greaves and Adair, all of the above-mentioned methods require large and expensive apparatus to dry enough plasma to consider the process practical. In addition, the use of chemical desiccants requires redrying of the desiccant each time it is used, an operating entailing per se considerable time and expense.

An apparatus for the drying of plasma from the frozen state by low temperature water vapor condensation in vacuo, previously described by us,[12] was based on the principles laid down by Bordas and d'Arsonval and on the work of Shackell,[3d] Harris,[3c] and Rogers.[3e] In the construction of the drying chamber, advantage was taken for proper heat distribution to the material being dried of copper split cylinders or shells enveloping each bottle. These shells, properly fastened together, formed also a desirable method of handling the bottles during the loading of the drying chamber, making the operation easy and rapid. In the construction of the condenser, we took advantage of details of construction pointed out by Reichel[5] and by Flosdorf and Mudd.[6] This condenser was externally cooled by the use of alcohol-carbon dioxide ice mixture.

Routine use of this apparatus pointed out that while the drying chamber proved fairly satisfactory, the condenser was rather crude in design and expensive in operation, particularly because of the cost of carbon dioxide ice and the necessity for almost continuous supervision. The presence of a secondary condenser made the apparatus difficult to handle and proper insulation an almost impossible task with the result that much of the low temperature produced by the carbon dioxide ice was wasted. The time of drying was three days or more.

An attempt was made to eliminate the use of dry ice by cooling the condenser by the use of a low temperature, two-stage (cascade) compressor using Freon-12 (or propane) and ethane as refrigerants and of a circulating brine system giving a condensing temperature of about minus 60° C. This apparatus proved too bulky, expensive, and unreliable in performance.*

Our early choice of low temperatures, in the neighborhood of minus 60° C. to minus 70° C. for the operation of this condenser as well as for the prefreezing of the plasma, was suggested by the generally held idea that such low temperatures were essential for the proper preservation of the product as well as for the proper functioning of the apparatus.[5, 6]

*Better two-stage compressors are built today, but they do not offer any practical advantage by the use of lower temperatures to offset the greater cost of purchase and maintenance.

DESCRIPTION OF NEW APPARATUS

The construction of a condenser with refrigerated coils within the condenser chamber was next attempted. Coils within the condenser are more efficient because of the excellent insulation provided by the high vacuum. This condenser was and is being used with a drying chamber similar to the one previously described.

The standard apparatus described here is capable of drying, in less than twenty-four hours, twenty-four lots of plasma each containing 300 c.c. in a 400 c.c. container. Thus the capacity is 7,200 c.c. in twenty-four hours. The residual moisture of plasma dried in this apparatus is consistently well below 1 per cent of the weight of the remaining material, assuring adequate preservation and rapid solution when the plasma is restored with solvent.*

It is to be noted that the time of drying depends upon several factors. The most important are as follows:

1. The thickness of the shell of the frozen material. A thinner shell dries much more rapidly than a thick one.

2. The amount of heat applied to the frozen material in the process of drying. The more heat applied, the more rapid is the process. Details of the application of heat will be elaborated upon separately.

3. The diameter of the neck of the bottle. The larger the opening, the more rapid the process within certain limits.

4. The type of protection used to maintain sterility. In our method sterility is adequately maintained by covering the individual bottles with a large cuff made from two layers of 40-mesh gauze. This offers some resistance to the flow of water vapor and, therefore, lengthens the time required for drying. It is felt, however, that this is a desirable precaution against contamination.

The complete apparatus described here is used for prefreezing (shell freezing) the plasma and for drying the plasma from the frozen state. It consists of four main parts: (1) a mechanical shelling apparatus, (2) a drying chamber with a water jacket, (3) a condenser with a mechanically refrigerated coil, and (4) a vacuum pump.

1. *Shelling Apparatus* (Fig. 2).—This consists of an insulated metal pan, containing the cooling coils and the apparatus for rotating the bottles containing the plasma.

The cooling coil is formed of a series of straight copper tubes. The two center tubes are shorter to make room for a propeller which circulates the cold alcohol. A plate type evaporator of suitable capacity may be used instead of coils. The shelling pan is connected with a reservoir for the alcohol. Before use, alcohol is pumped into the shelling pan. At the end of the process, the alcohol is returned to the reservoir by gravity. The shelling pan contains a mechanical device, capable of turning twelve bottles at the rate of one-half to one rotation per minute. The arrangement of the rotating wheels is so that the bottles will be immersed in the cooled alcohol about 12 mm.

*The standard apparatus here described is constructed by the Precision Scientific Company of Chicago.

2. *Drying Chamber* (Fig. 3).—The drying chamber consists of a heavy brass cylinder (22.5 cm. in diameter and 70 cm. high) surrounded by a water jacket. It is closed on top by a heavy steel lid, which rests on a rubber gasket. The lid may be tightened by threaded lugs and wing nuts. The opening for the connection with the condenser is in the middle of the base of the drying chamber. This location constitutes an essential feature of the apparatus. It is flush with the base itself and is 5 cm. (2 inches) in diameter. The connection with the condenser is made by a short copper pipe, 5 cm. (2 inches) in diameter.

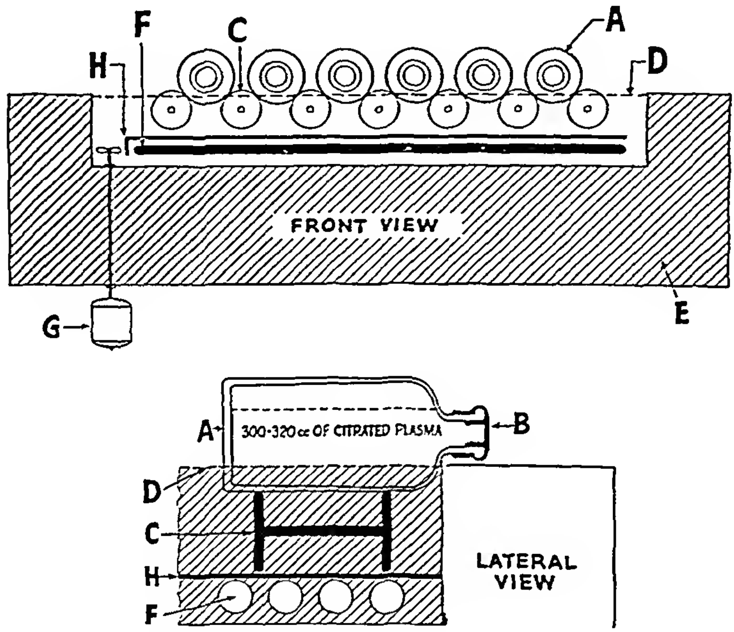


Fig. 2.—Apparatus for shell freezing with circulator for refrigerated alcohol (schematic). *A*, 400 c.c. bottle; *B*, rubber stopper; *C*, rubber-tired rollers; *D*, refrigerated alcohol level; *E*, insulated metal pan; *F*, evaporator coils; *G*, circulator; *H*, baffle board.

The drying chamber is surrounded by a water jacket formed by a cylinder of rustproof metal fixed to the flange of the inner chamber. The water jacket is 30 cm. (12 inches) in diameter and 90 cm. (36 inches) overall in length. It is provided with an electrical heater, fitted to the bottom and capable of maintaining the water at any desired temperature up to 80° C. This heater is controlled by a thermostat and relay. The temperature may be adjusted by setting the thermostat. The water bath is connected by pipes to sources of hot and cold water.

The dry chamber is fitted with four copper baskets. Each basket is composed of an outer split cylinder of 24 oz. copper approximately 22.5 cm. in diameter (9 inches) and 20 cm. in height (7⅞ inches). The lower border is turned in 12 mm. (½ inch). This strengthens the cylinder and holds the plasma bottles in place. This large cylinder contains a series of six smaller split copper cylinders welded to the inner surface. These are 11.8 cm. high (4⅝ inches) and are of the proper diameter to grip the plasma bottle tightly. The basket is completed by a transverse bar and springs, which maintain proper expansion

of the outer cylinder, and by a series of copper wedges, which assist in increasing heat condution as well as the contact of the copper shells with the bottles.

A recording thermometer is provided to determine the temperature of one of the copper shells of the lower basket. It has a range from minus 10° C. to plus 80° C. The bulb of the thermometer is fixed to the bottom of the drying chamber and is in direct contact with two of the copper cylinders. Its reading is affected by the temperature of the bottle of plasma in the copper cylinders. A dial thermometer shows the temperature of the water bath. When the temperature shown by these two thermometers remains within 42° C. for two hours or more, the plasma may be considered dry.

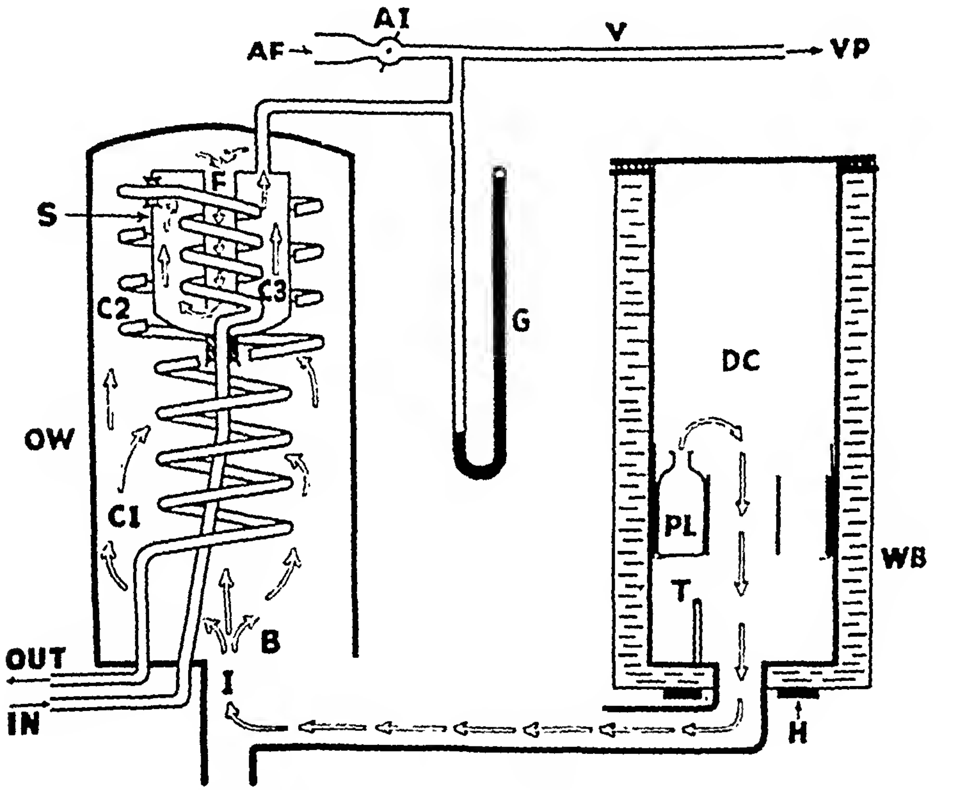


Fig. 3.—Apparatus for drying of plasma (semischematic).

IN, Inlet of evaporator; *OUT*, o[illegible] *OW*, wall of condenser chamber; *C1-C2-C3*, portions of the evaporator ([illegible] *I*, inlet for water vapor; *F*, funnel-like opening conveying water vapors f[illegible] denser chamber to the coldest portion of the evaporator coil, *C3*; *B*, base of the condenser chamber; *G*, vacuum gauge; *AF*, air filter; *AI*, air inlet valve; *V*, vacuum line; *VP*, vacuum pump; *H*, 750 Watt heater; *WB*, water bath; *DC*, drying chamber; *Pl*, plasma; *S*, shield of condenser; *T*, bulb of recording thermometer.

One of the difficulties in the drying of plasma from the frozen state is the proper application of heat to the frozen plasma to counteract the cooling effect of rapid evaporation. If no heat is applied, the process will require several days, while properly controlled heating reduces the time to less than twenty-four hours. Heat from the water bath around the drying chamber is conducted to the plasma very satisfactorily by means of the copper baskets just described.

3. *Condenser* (Fig. 3).—In the construction of the condenser there are two essential features:

1. The large water vapor inlet is located in the lowest part of the condensing chamber.

2. The refrigerated coils are so arranged that by means of properly placed cylindrical shields, the water vapor is made to travel a long distance and come in contact with a large condensing surface. The last portion of the coil with which the vapor comes in contact is the coldest. This assures condensation of all of the water vapor and prevents any water from mixing with the oil of the vacuum pump.

The water vapor enters the condenser from the bottom. This is very important, because it allows slower diffusion of the water vapor and allows maximal condensation on the first set of coils with which the vapor comes in contact. Under ordinary conditions, the greatest portion of the vapor is condensed on Coil C1. Some vapor escapes Coil C1 and reaches Coil C2. Here the vapor travels through a rather narrow space between the wall of the condenser and the cylindrical copper shield S placed inside of Coil C2. If a trace of water vapor escapes Coil C2, it must travel through a narrow funnellike tube (F) to reach Coil C3 which is enclosed in a copper box. The vacuum pump is connected to the condenser by means of an opening in the top of this box. The great bulk of the water vapor is condensed on Coil C1, a smaller amount on Coil C2, and only a trace on Coil C3 (Fig. 4). No water escapes to the vacuum pump. This has been determined by placing a copper U-tube 5 cm. in diameter in the vacuum line V during experimental runs. The U-tube was immersed in a bath of carbon dioxide ice and alcohol maintaining a temperature of minus 70° C. to minus 72° C. At the end of the runs no ice was found in the U-tube.

A Servel F-12, four-cylinder, ¾ h.p. compressor with dehydrator and oil separator is very satisfactory for cooling the coils in the shelling pan and in the condenser. The shelling pan and the condenser may be cooled simultaneously or individually. The F-12 gas should be pumped out of the coils before heat is applied in the condenser to melt the ice at the end of each run. The coils in the condenser will be maintained from minus 35° C. to minus 40° C. without a load, and from minus 30° C. to minus 35° C. while the drying is being accomplished. Occasionally during the period of maximal condensation, the temperature may be a little higher.

The condenser chamber itself is made of copper sufficiently strong to withstand a high vacuum. The total length of the refrigerated coil is 13 m. (44 feet), and the inner diameter 1.56 mm. (⅝ inch). A dial thermometer shows the temperature of the "In" coil of the condenser. This greatly assists in checking proper operation of the refrigerating compressor. An electric heater is provided in the condenser to melt the ice from the coils rapidly at the end of each drying run. The melted ice is then drained from the condenser through the opening in the bottom.

The copper box (S of Fig. 3) is so constructed that the ice formed on Coil C3 will drain out once it is melted. This is accomplished by means of a self-sealing valve surrounding the "In" coil as it enters the box. The valve consists of a short length of copper tube insulated from the rest of the box and approx-

imately 2 mm. larger in diameter than the refrigerated coil passing through it. When the drying process is initiated, water vapor very soon freezes around the refrigerated coil passing through the valve, and so seals it. When the ice is melted, the valve is open and allows water from Coil C3 to drain from the condenser.

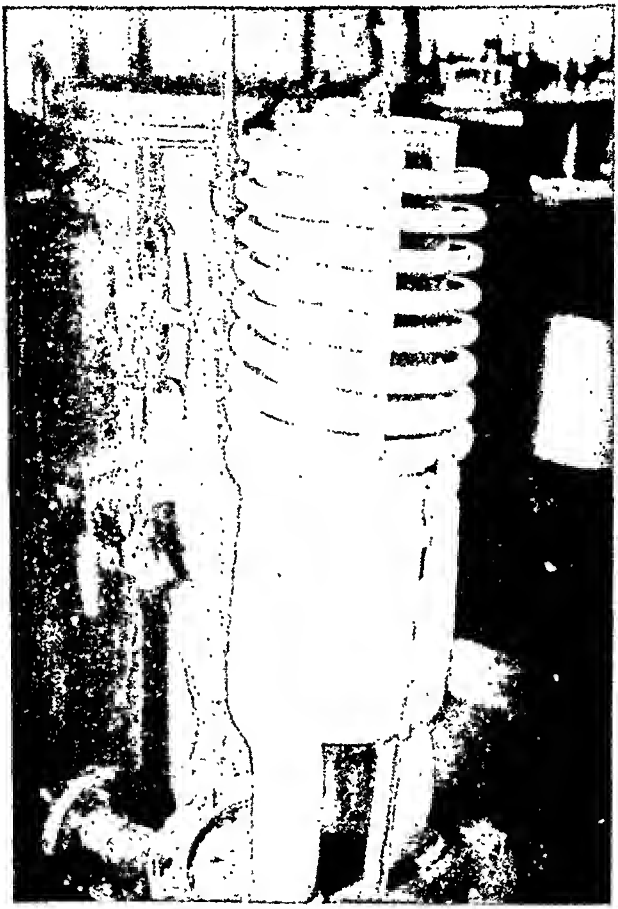


Fig. 4.—Ice formation on the condensing coil after a standard run (about 7200 c.c.)

4. *Vacuum Pump and Gauge.*—The large Welch Duo-Seal or the Cenco-Megavac with ½ h.p. motor has proved adequate. The pump must have a capacity sufficient to evacuate the system to a pressure of less than 2500 microns in ten minutes and 500 microns in less than fifteen minutes with the temperature of the condensing coils at minus 30° C. to minus 40° C.

A Pirani type vacuum gauge, such as the Truvac Gauge, Model 12, is satisfactory. It should be graduated from 2500 microns to 0 microns. A MacLeod type mercury gauge is also satisfactory. It is desirable to have, in addition, a rough dial type vacuum gauge, reading from atmospheric pressure to zero, to be able at a glance to watch the initial rate of evacuation of the system when a drying run is being started. This ascertains, in the first minute or so, that all outlets have been properly closed.

The entire apparatus is compactly assembled in a single cabinet and all switches, valves and recording dials and instruments are placed on a single panel (Fig. 5).

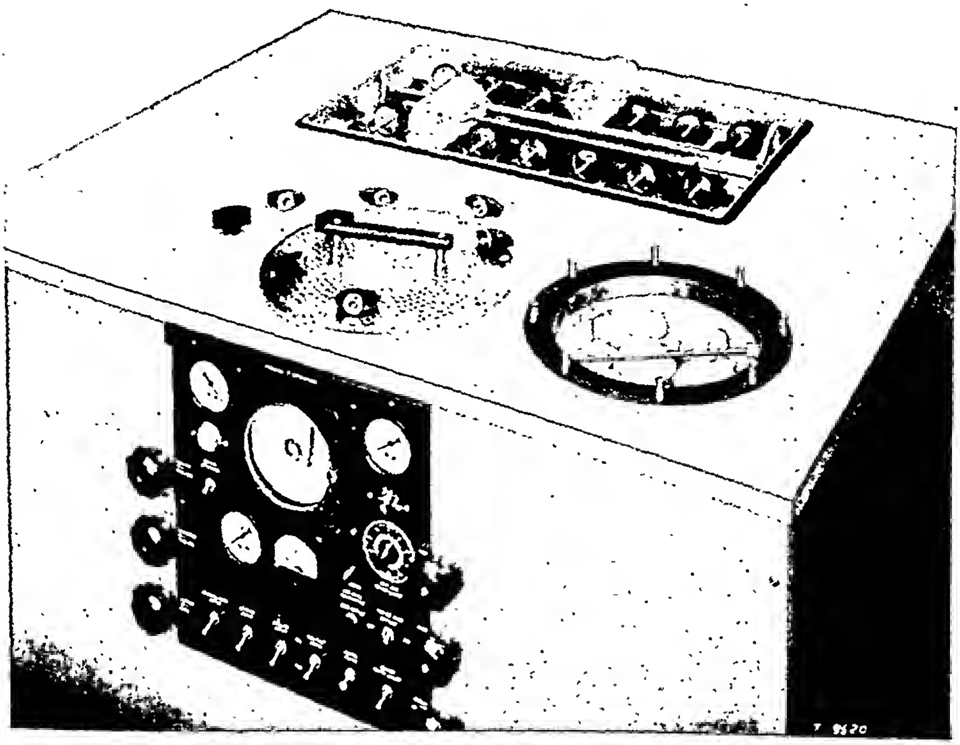


Fig. 5.—Standard shell freezing and drying apparatus. In the rear can be seen the shelling pan; in the right anterior corner the drying chamber, and in the middle front the panel board.

MECHANICAL SHELL FREEZING AT MINUS 20° C. TO MINUS 35° C.

We had previously found that the choice of low temperatures in the neighborhood of minus 60° C. to minus 70° C. for rapid prefreezing of plasma does not offer any practical advantage. A mechanical apparatus which gave good results[1a] was developed for shell freezing at minus 40° C. It was found, however, that shell freezing at temperatures higher than minus 40° C. was not possible with continuous rotation, because plasma would become supercooled at minus 6° C. to minus 12° C., and would then "snap" freeze, with slushing and formation of an irregular shell, with large lumps which greatly interfered with drying later on.

In attempting to shell freeze plasma at temperatures between minus 20° C. and minus 35° C., it was noted that if early initial local freezing occurred in contact with the inner surface of the bottle, shell freezing continued thereafter without lumping. It appeared necessary in the course of experimental work to produce a relatively rapid cooling of plasma to about plus 10° C. from room temperature followed by local freezing, to avoid supercooling of the entire mass and slushing following "snap" freezing. This is accomplished with the fol-

lowing technique: The plasma bottle is rotated slowly (½ to 1 r.p.m.) with 12 mm. immersion in alcohol cooled to minus 30° C. to minus 35° C. for a period sufficiently long to bring the temperature down to about plus 10° C. The length of time required for this operation depends chiefly on: (a) the temperature of plasma, and (b) the temperature of the brine. It varies from three to ten minutes. With plasma at plus 25° C. and the brine at minus 30° C., about ten minutes are sufficient. After the period of cooling, the rotation is stopped long enough to cause the lower portion of the plasma to "snap" freeze to a depth of about 3 to 4 mm. This requires two to four minutes. The phenomenon is, as a rule, clearly visible. It is important to remember that during this period of time, the bottle of plasma must not be disturbed, and that the depth of immersion of the bottle in the cooled alcohol must be 10 to 12 mm. only. Occasionally, and for no apparent reason, one bottle may take considerably longer to show initial "snap" freezing. It must not be disturbed until "snap" freezing has occurred. It is desirable to obtain a temperature of minus 35° C. or a little lower before placing the bottles in the shelling pan.

When the local "snap" freezing has occurred in all of the bottles, the rotating motion is resumed, and shell freezing occurs at a regular rate and very evenly. With a temperature varying between minus 35° C. and minus 25° C., it requires one hour and fifteen minutes to one hour and twenty-five minutes for complete shell freezing of 300 c.c. of plasma. The quality of the material thus frozen is practically identical with that obtained from shell freezing at minus 70° C. The material thus obtained is readily recognized as it shows a longitudinal portion of microcrystalline, homogeneous aspect, lighter in color, and about 4 to 5 cm. wide and 3 to 4 mm. thick. The remaining portion shows a macrocrystalline structure. This aspect is clearly retained after drying from the frozen state (Fig. 6).

When the plasma is shelled, the bottles are placed in a storage cabinet at minus 20° C. to minus 30° C. to cool. It takes about four hours for the frozen material to cool from about 0° C. to minus 20° C. The plasma thus shelled can remain in cold storage for an indefinite period of time.[15]

About one hour before initiating the drying process, the copper baskets are placed in the low temperature cabinet to cool. At the same time, the bottles of shell-frozen plasma are removed, one by one, from the storage cabinet and, under aseptic precautions, the rubber stoppers are replaced by large sterile gauze cuffs formed of two layers of 40-mesh gauze. The bottles are now placed in the copper baskets, and allowed to cool for about one-half hour at minus 20° C. to minus 30° C.

TESTING AND OPERATION OF THE APPARATUS

Before each run the apparatus should be tested to be sure it will produce a satisfactory vacuum and low temperature. The drying chamber and the inlet valve are closed, and the compressor is started and allowed to run until the thermometer on the condensing coil registers minus 30° C. or less. The vacuum pump may then be started and a vacuum of 500 microns (Truvac gauge) or less must be obtained in fifteen minutes or less. If this is accomplished, the vacuum pump is shut down, the vacuum is released, and the drying chamber opened.

The copper baskets containing the shell-frozen plasma are rapidly placed in the chamber, the lid is closed, and the vacuum pump started. This operation can readily be accomplished in about two minutes. A pressure of 500 microns or less should be attained in fifteen minutes or less to avoid danger of thawing. The water in the water jacket of the drying chamber must be cool, not over 25° C.

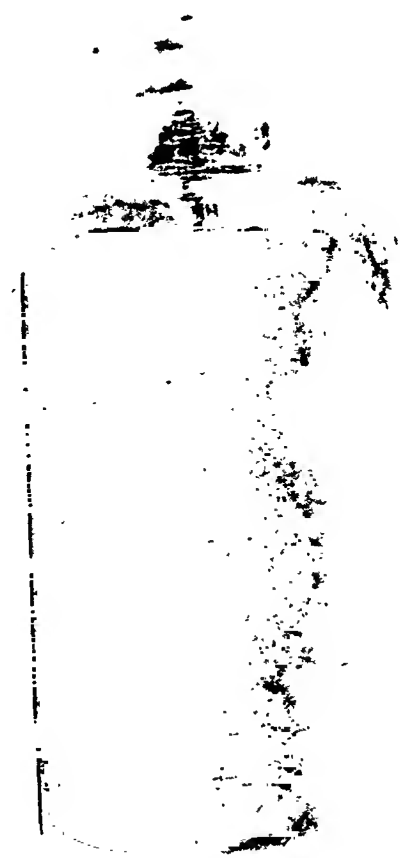


Fig. 6.—Bottle of dried plasma showing on the left homogeneous aspect corresponding to the portion of plasma "snap" frozen, and on the right the gross crystalline structure prevailing in the remaining portion.

The operation is allowed to proceed for one hour. The heater of the water jacket is then turned on with the thermostat set at plus 50° C. Drying is allowed to proceed until the thermometer recording the temperature of the plasma has been for two hours or more within two degrees of the temperature of the water in the water jacket. The vacuum pump is then stopped, and filtered air is allowed to enter slowly through the inlet valve. This air passes through the condenser before it enters the drying chamber and thus is dried. The opening of the inlet valve is covered with 40 to 50 layers of gauze to effect a good filtration of air, sufficient per se to minimize danger of contamination. The air should be allowed to enter slowly, taking about five minutes for the entire operation. The copper baskets are removed one at a time, and the bottles promptly stoppered.[12] The bottles are then evacuated, and the rubber stoppers are covered

with gel caps. This method of closure is sufficient for preservation up to three years. If the preservation is to be for longer periods of time, the material is vacuum-sealed in tin cans.[14]

The time of drying with a continuous temperature of 50° C. in the water jacket is less than eighteen hours. When it is desirable to reduce the drying time, the temperature of the water jacket may be increased up to 80° C. for a period of time.

The dried plasma obtained with the apparatus and technique outlined above is a light, porous material, with a distinct crystalline structure, and a volume slightly less than that occupied by the frozen plasma. It may be readily reduced to a fluffy powder by shaking, in which case it occupies about one-third of the original volume. The powder thus obtained is readily transferred to another receptacle for purpose of residual moisture determination or of combining the content of two or more bottles. The color of the dried plasma is light amber, when the original liquid plasma had a hemoglobin content of less than 5 mg. It is very soluble in water. When solvent is introduced by vacuum suction, dried plasma containing 17.5 Gm. of plasma proteins (the equivalent of about 300 c.c. of fresh citrated plasma) dissolves entirely in 250 c.c. of distilled water or 0.1 per cent citric acid solution in less than one minute, including the time consumed by the introduction of the solvent.

The resulting fluid is stable at room temperature for at least several days; it has a maximal content of complement, and a maximal content of prothrombin if regenerated with 0.1 per cent citric acid solution.[17] The use intravenously of properly prepared material causes no untoward reactions, save an occasional (less than 1 per cent) urticarial reaction.

The size of the apparatus here described is considered best for the purpose intended, namely, for large hospitals to dry plasma for their own use and for neighboring institutions. Both smaller (1800 c.c. daily capacity) and larger (21,600 c.c. daily capacity) units have been successfully operated, however. When larger units for commercial operation are built, it is desirable to institute automatic temperature control, for minimum drying time with the least technical supervision, by the installation of a cam-type combination thermoregulator and recording apparatus, such as the one furnished by the Foxboro Company, Mass.

In addition to human plasma, serum, guinea pig complement, bacterial cultures, milk, many fruit juices, culture media, and even meats and other products have been dried with the machine here described with excellent preservation of all essential properties including flavor and vitamin content.

The advantages of the apparatus here described over other apparatus are:

1. Both prefreezing in shell form and drying from the frozen state are accomplished by employing the same economical source of low temperature.

2. The apparatus is very simple in operation and almost entirely automatic.

3. It yields constantly a product in every way satisfying the requirements set forth by the National Institute of Health.

4. The time of drying is considerably shorter than that obtained with other methods.

5. The total cost of operation per unit is very small.

REFERENCES

1. (a) Strumia, Max M., McGraw, John J., and Reichel, John: Preparation and Preservation of Human Plasma. III—Freezing of Plasma and Preservation in the Frozen State, Am. J. Clin. Path. 11: 388, 1941.
   (b) Strumia, Max M. and McGraw, John J.: Frozen and Dried Plasma for Civil and Military Use, J. A. M. A. 116: 2378, 1941.
2. Harkins, H. N.: Recent Advances in the Study and Management of Traumatic Shock, Surgery 9: 607, 1941.
3. (a) Vansteenberghe, P.: Procede de conservation du virus rabique a l'etat sec, Compt. rend. Soc. de biol. 4: 1646, 1903.
   (b) Bordas, F., and d'Arsonval: Les basses temperatures et l'analyse chimique, Compt. rend. Acad. d. sc. 142: 1058, 1906.
   (c) 1. Harris, D. E.: The Properties of Desiccated Rabies Virus and Its Use in Antirabic Immunization, J. Infect. Dis. 10: 369, 1912.
   2. Harris, D. E.: Recherches sur les properties du Virus Rabique conserve a l'etat sec, Ann. Inst. Pasteur 26: 732, 1912.
   (d) Shackell, L. F.: An Improved Method of Desiccation, With Some Applications to Biological Problems, Am. J. Physiol. 24: 325, 1909.
   (e) Rogers, L. R.: The Preparation of Dried Cultures, J. Infect. Dis. 14: 100, 1914.
   (f) Elser, W. J.: U. S. Patent 1970956, 1934.
   Elser, W. J., Thomas, B. A., and Steffen, G. I.: Desiccation of Sera and Other Biological Products (Including Micro-organisms) in the Frozen State With Preservation of Original Qualities of the Products So Treated, J. Immunol. 28: 433, 1935.
4. Folsom, T. R.: An Effective Technic for Desiccating Plasma in Useful Quantities: The Sterile Unit Desiccator, War Med. 1: 342, 1941.
5. Reichel, J.: U. S. Patent Re 20969, 2066302.
6. Flosdorf, E. W., and Mudd, S.: Procedure and Apparatus for Preservation in "Lyophile" Form of Serum and Other Biological Substances, J. Immunol. 29: 389, 1935.
7. Flosdorf, E. W., and Mudd, S.: Improved Procedure and Apparatus for Preservation of Sera, Micro-organisms and Other Substances—Cryochem Process, J. Immunol. 34: 469, 1938.
8. Hill, J. M., and Pfeiffer, D. C.: A New and Economical Desiccating Process Particularly Suitable for the Preparation of Concentrated Plasma or Serum for Intravenous Use: The Adtevac Process, Ann. Int. Med. 14: 201, 1940.
9. Greaves, R. I. N., and Adair, M. E.: High Vacuum Condensation Drying of Proteins From the Frozen State, J. Hyg. 39: 413, 1939.
10. Flosdorf, E. W., and Mudd, S.: The Desivac Process for Drying From the Frozen State, J. A. M. A. 115: 1095, 1940.
11. Ely Lilly Company, Indianapolis, Indiana: Personal communication (1940).
12. Strumia, Max M., McGraw, John J., and Reichel, John: The Preparation and Preservation of Human Plasma—IV Drying of Plasma From the Frozen State by Low Temperature Condensation in Vacuo, Am. J. Clin. Path. 11: 480, 1941.
13. Strumia, Max M., and McGraw, John J.: Blood Plasma, Its Place in the Practice of Medicine, With Special Consideration to the Problems of Preservation, J. A. M. A. 118: 427, 1942.
14. Strumia, Max M., Newhouser, Lloyd R., Kendrick, Douglas, B., Jr., and McGraw, John J.: Development of Equipment for Administration of Dried Plasma in the Armed Forces, War Med. 2: 102, 1942.
15. Strumia, Max M.: Preservation of Prothrombin in Dried Plasma, J. A. M. A. 119: 710, 1942.
16. Hoxworth, P. I.: Personal communication.
17. James, W. E.: Personal communication.

A NEW FIBRINOLYTIC METHOD FOR THE ASSAY OF PROTEASES AND ITS APPLICATION TO AN ANALYSIS OF COAGULATION PHENOMENA IN RABBIT BLOOD*

JOHN H. FERGUSON, M.D., ANN ARBOR, MICH.

SINCE 1939, this laboratory has been contributing evidence for a new theory[1] of blood-clotting, based upon the dominant role of serum-tryptase, a proteolytic enzyme, long recognized by biochemists (Oppenheimer[2]). Even in the complete absence of formed elements, this plasma enzyme normally performs actions which we are able to duplicate with *crystalline* (pancreatic) trypsin, in vitro. These actions are (1) thromboplastic conversion of prothrombin to thrombin[1b, 3] (whence the designation "thromboplastic enzyme"[4]), (2) digestion of prothrombin[5] and thrombin[6] (whence the term "progressive" antithrombin to distinguish the latter from "immediate," neutralizing and reversible[7] inhibition of thrombin, for example by heparin and its albumin-fraction "cofactor"), (3) lysis of fibrinogen and fibrin[8] (whence the term "fibrinolysin" is superfluous). Clot retraction is probably just the initial phase of tryptic digestion of the clot, and natural fibrinolysis plays an important role in the resolution of (blood) clots in vivo.[8] Ferguson's claim that a plasma deficiency of available tryptase is the true explanation of the coagulation delay in *hemophilia*,[9] has been confirmed by Feissly,[10] with the aid of a gelatine liquefaction technique. Continued search for a method whereby this and other problems relating to the tryptase factor could be put to a quantitative test, has yielded a new assay technique, as follows:

ASSAY OF TRYPSIN BY FIBRINOLYSIS

(*Relative Turbidimetry With the Photoelectric Colorimeter*). *Method.*[11]—Varying amounts of trypsin are added to alkaline (buffered) mixtures of fibrinogen and thrombin and lysis of the clots formed is timed by the relative positions of the curves charted with the aid of the Evelyn apparatus.

Reagents.—The test requires a stable fibrinogen and a stable thrombin. Citrated blood plasma contains a natural lytic factor (serum tryptase), but this apparently deteriorates on keeping. Hence, the *fibrinogen* (F) is routinely prepared by three precipitations with $(NH_4)_2SO_4$ (¼ sat.), from refiltered (dog) plasma, several days old. The preparation is suitable if it shows no visible lysis (in the control) during the course of the experiment. An excellent stable *thrombin* (T_0) is obtained by a 1:100 dilution of Parfentjev's[12] "rabbit clotting globulin" (Lederle Laboratories). In an optimal series of *trypsin* dilutions, the highest enzyme concentration used causes fibrinolysis in one and one-half to two hours and the weakest in six to twelve hours (a matter of convenience). The strongest typsin should not significantly interfere with clot formation. All reagents contain 0.9 per cent NaCl (plus thymol) and

*From the Department of Pharmacology, Medical School, University of Michigan.
Received for publication, Sept. 21, 1942.

the fibrinogen is buffered with barbiturate,[6] borate, or other suitable buffer. A routine pH of 7.5-7.75 is sufficiently alkaline for the enzyme action, without increasing the translucency (impairing turbidity) to the extent seen with unduly alkaline mixtures. The following amounts of reagents are mixed in the order cited: 5 c.c. *buffered* fibrinogen + 1 c.c. saline (= control for experiments on inhibitor or other agents it is desired to study) + 3 c.c. thrombin + 1 c.c. trypsin. In order to minimize lysis of the fibrinogen and thrombin, the enzyme is added five to ten seconds before the onset of clotting.

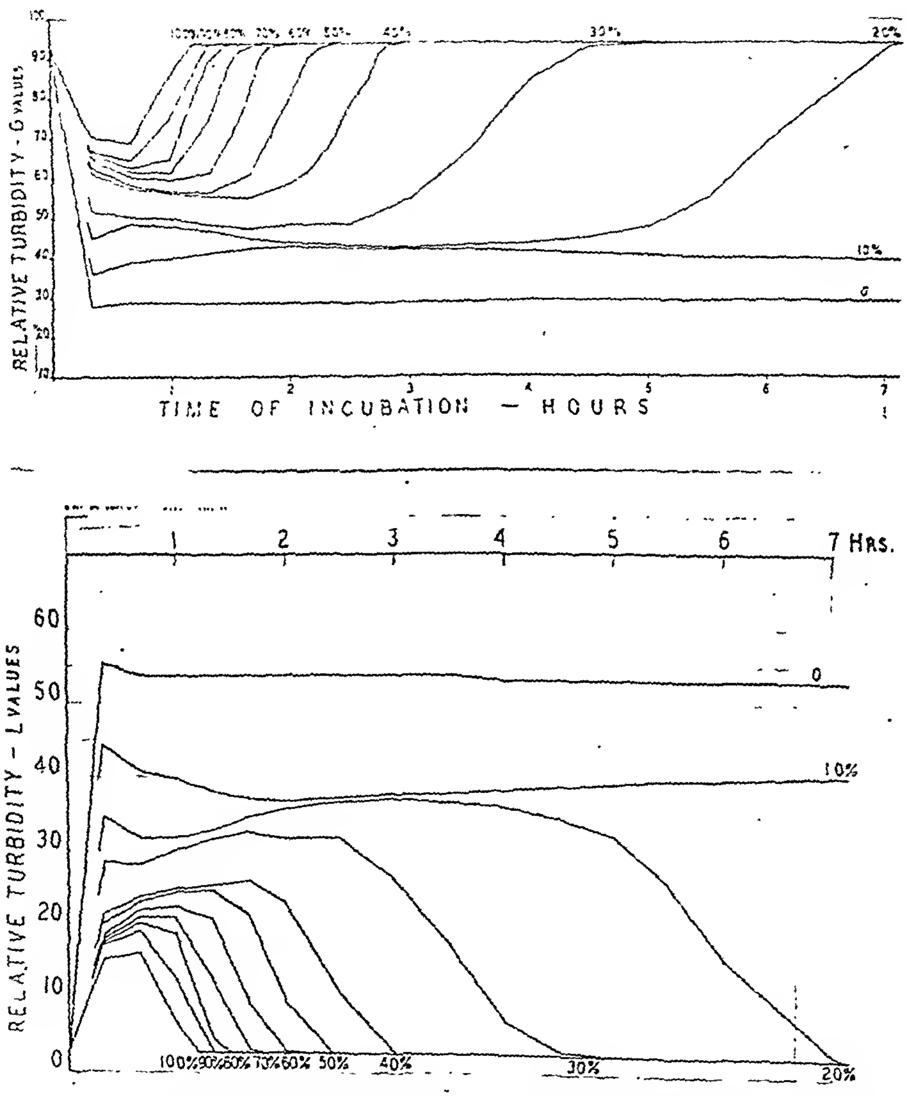


Fig. 1.—Assay of crystalline trypsin (standard X.T.) by fibrinolysis. Relative turbidimetry with the Evelyn photoelectric colorimeter. 1·10 vol. of 1:1000 X.T. = 100%. Temp. = 22° C., $pH = 7.5$.

Data.—The data presented graphically in Fig. 1 form a typical reference series on a crystalline trypsin preparation, 1 mg. of which analyzed 4.24×10^{-3} [T.U.]m, by the Anson and Mirsky method.[13] Ten per cent fractions of a stock (100 per cent = 1:4000) were used in one-tenth vol. (v. supra), hence the *final* concentrations vary from 1:40,000 down to 1:400,000. The curve for 1:400,000

(10 per cent) is incomplete since fibrinolysis in this case required 15 to 16 hours, as compared with 7 hours, 5 hours, 3 hours, 145 minutes, 130 minutes, 110 minutes, 90 minutes, 80 minutes, 72 minutes, for the 20 per cent to 100 per cent, respectively. The control was completely unaffected in 24 to 48 hours. Temp. = 22° C., pH = 7.5 (checked in all tubes, with glass electrode, at completion of fibrinolysis). Clotting-time (C.T.) = 25 seconds: the same in the control and all the enzyme-containing mixtures.

Instrument data.—Green filter (510). Galvanometer (G) values: (1) Blank (10 c.c. saline) = 1000; (2) initial reading (all tubes) = 930; (3) final reading (all tubes, except control) = 910.

The slight persistent turbidity is due to a very faint opalescence, probably associated with traces of lipids or thrombin impurities which are still present after fibrinolysis.

Comment.—The absence of C.T. change is evidence that the thrombin and fibrinogen are not significantly attacked by the trypsin concentrations used. However, the relative turbidity is progressively lessened as the enzyme strength is increased (see Fig. 1) and this is probably a more sensitive indication of a minor degree of fibrinogen destruction. Such fibrinogenolysis as may occur enhances the sensitivity of the turbidimetric test, but lessens the significance of the actual values. It is not the shape of the curves which is significant, but their relative positions along the time axis. A simple charting of galvanometer scale readings (G) serves the purpose of *timing* the fibrinolysis just as satisfactorily as the corresponding "photometric densities" (L), which require computation by the formula L = 2 – log G. Both methods are illustrated in Fig. 1. The steep terminations of the curves for the various mixtures are clearly separated by marked time differences. Hence, the strength of any "unknown" solution of similarly-acting lytic agent (suitably diluted) can be assayed from the position of its end-curve with reference to those for the standard trypsins. A good commercial trypsin of known potency is just as serviceable as the crystalline enzyme.* The 10 per cent "limit of error" is extremely liberal, particularly with the longer-acting (i. e. weaker) trypsins.

In preliminary testing for suitable dilution range, simple visual timing of fibrinolysis is adequate and the photometer can also be dispensed with if the "lytic times" are sufficiently long and well separated. Turbidimetry is invaluable, however, for enhancing the reliability of observation and permitting assays to be made with the greatest possible accuracy in the shorter time intervals. The absolute practical sensitivity of the fibrinolytic assay is of the order of 1:1,000,000 final crystalline trypsin ($=4.24 \times 10^{-6}$ $[\mathrm{T.U.}]_{\mathrm{Hb}}$), which makes the method especially applicable to quantities of enzyme too small to be assayed by all but the best (Anson and Mirsky[13]) proteolytic methods. It offers three improvements over the method used for bacterial "fibrinolysin" by Christensen:[14] (1) standard reference enzyme, (2) greater accuracy of the photoelectric timing, (3) an absolutely stable (tryptase-free) fibrinogen is not essential. A trace (0.1 per cent) of citrate is not objectionable, but tryptase inhibitors must be absent.

*Further detail as to standard "units" and reagents is given in a note to Federation Proc. (1943). A Newer Method, by Lysis of Fibrinogen, Proc. Soc. Exper. Biol. & Med. 52: 243, 1943.

Effects of Varying Fibrinogen Concentration. Method.—Five strengths of a fibrinogen solution (v. supra), which yielded 200 mg. of *fibrin* per 100 c.c. (dry weight, by a modified Gram method), were clotted by T_6 (as above) in the presence of 1/10 volume of 1:4000, 1:8000, 1:16000, 1:32000 N.T., along with a control (without trypsin) for each series. Table I summarizes both the clotting-times and the "lytic times," determined with the aid of the photoelectric colorimeter.

Comment.—As expected, the rate of fibrinolysis is speeded up by lowering the substrate (fibrin) concentration. The clotting-times indicate that the 0.2 per cent fibrinogen was a little too strong for optimal clotting and gave slowest lysis with both strongest and weakest enzyme concentration. The 0.0125 per cent fibrinogen was too weak, giving longer clotting-time (hence favoring fibrinogenolysis by the added enzyme) and insufficient turbidity. Nevertheless, the control remained stable for three days and the turbidimetric timing of the lysis seemed satisfactory. It was particularly noted that the weaker fibrins gave quicker lysis with both the strongest and weakest enzyme concentrations. The facts suggest a trace of *natural inhibitor* in the fibrinogen. With intermediate enzyme concentration (final conc. = 1:80,000, 1:160,000) the varying fibrinogen produced only minor differences in the lytic times. It may be concluded that a fibrinogen concentration of 0.05 to 0.1 per cent is optimal for the trypsin assay. With trypsin concentrations below 1:300,000 (final), somewhat weaker fibrinogens may be advocated.

Effects of Temperature and pH.—Our experience confirms the information given by Christensen, for bacterial "fibrinolysins," and the data need not be presented in detail. We find it convenient to carry out the lytic tests at room temperature (20° to 25° C.) and a thermostat water-bath is indicated only for timing lysis occurring more rapidly than two hours. Tryspin has a broad zone of optimal pH (8 to 9. Northrop) and considerable activity is retained well into the acid side of neutrality. The fibrinolytic method is adapted to the assay of any proteolytic enzymes which are active between pH = 6.5 and 8.5. Too great alkalinity lessens the "photometric density" of the fibrin gel, leading us to prefer a pH = 7.5 – 7.75 as the practical optimum for the method, albeit this is about the limit of usefulness of the barbiturate buffer recommended (since barbituric acid tends to precipitate from the buffer mixture).

TABLE I

EFFECTS OF VARYING FIBRINOGEN CONCENTRATION ON ASSAY OF TRYPSIN BY FIBRINOLYSIS. "LYTIC TIMES" AT 25° C., pH = 7.5

FIBRINOGEN CONC. (%)	0.2	0.1	0.05	0.025	0.0125
Trypsin conc. (final)					
1:40,000	75 min.	60 min.	45 min.	40 min.	35 min.
1:80,000	2 hr.	2½ hr.	1¾ hr.	1½ hr.	1¼ hr.
1:160,000	3½ hr.	4 hr.	3 hr.	3 hr.	2¼ hr.
1:320,000	9 hr.	7½ hr.	5 hr.	5 hr.	3½ hr.
0*	∞	∞	∞	∞	∞
Clotting-times	30 sec.	25 sec.	25 sec.	26 sec.	55 sec.

*None of the controls showed any lysis in 3 days. The clotting-times are for the controls, but were not significantly modified by the addition of enzyme in the manner specified.

Use With Other Proteases.—The new method obviously suggests application to the study of all "tryptases" (Oppenheimer[2]) including *those* of plasma, leucocyte (and platelet), and tissue origin; also bacterial proteases and the

proteases in snake venoms (which may possibly have a bacterial origin), and papain. It is an easy way to control steps in the purification of such enzymes and their behavior with respect to small changes in pH, salt concentration, action of inhibitors, etc.

SERUM-TRYPTASE AND BLOOD-CLOTTING IN THE RABBIT

The following materials were tested: A, 10 c.c. citrated (0.38 per cent) rabbit plasma, diluted to routine volume (25 c.c.) with thymol-saline; B, same (1:2 dilution) citrated plasma clotted by *excess* of $CaCl_2$, the fibrin being removed by whipping with a glass rod, leaving the clear serum for the chloroform treatment (infra); C, (1:2) citrated plasma, clotted by 2 *c.c.* enzyme-free thrombin (1:100 dilution of Parfentjev's rabbit globulin), the fibrin likewise being removed; D, the fibrin from C, washed thoroughly in three changes of saline and resuspended in routine volume (25 c.c.) of saline; E, ten-hour-old serum from clotted whole blood. In order to remove trypsin-inhibitors (which will not be considered at this time), we employed the old method of Nolf, et al. (Tagnon[15]), shaking with one-tenth volume of chloroform. The (50 c.c.) bottles were centrifuged after three-quarters of an hour, the supernatant being left in contact with the $CHCl_3$ and samples removed 1, 6, 24 hours from start of treatment. Clotting-tests and controlled fibrinolytic studies (routine trypsin-assay method) are summarized in Table II.

TABLE II

CLOTTING AND FIBRINOLYTIC TESTS ON RABBIT PLASMA MATERIALS

		CHLOROFORM TREATMENT			CLOT RETN.	LYSIS OF CLOT	LYTIC TIME (TRYPTASE TEST)
		(A) 1 HR.	(B) 6 HR.	(C) 24 HR.			
A	Plasma (citrated)	0 (24 hr.)	27 min. (+)	36 min. (+)	*none*	*none*	>4 days
B	Serum (excess Ca)	135 sec. (++)	40 sec. (++)	20 sec. (++)	3½ hr.	3-4 da.	40 hr.
C	Serum (Thrombic)	1 hr. (+)	0 (24 hr.)	2 hr. (+)	2nd day	3-4 da.	29 hr.
D	Fibrin (from C.)	51 min. (+)	12 min. (+)	58 min. (+)	2nd day	3-4 da.	20 hr.
E	Ten-hour serum (whole bld.)*	24 min. (++)	–	–	none	none	>4 days
F	Control (F + T_0)	35 sec. (++)	–	–	40 hr.	48 hr.	48 hr.

*0.1 c.c. serum + 1.0 F. A similar test with fresh (10 hr.) untreated serum clotted in 28 min.

TRYPTASE TESTS

Room temperature = 23° C. ± 2° C. pH = 7.75 (barbiturate buffer). 1 c.c. test-material in 10 c.c. fibrinogen-citrate (0.03 per cent)-buffer mixture. Standard trypsins: X.T. (v. supra) — 1:500,000; 1:1,000,000; 1:2,000,000 (final concentration) gave "lytic times" of 12½, 18, 24 hours, respectively, while the control (F) was stable for two days. Tests were made both with

the one-hour and the 24-hour samples, the latter with a weaker (1:3) fibrinogen. Except for a very slight increase in enzyme activity in *B* in the 24-hour, as compared with the one-hour sample, the data (including standards) were so similar that only the tests with the one-hour materials will be cited (Table II).

Data.—*A* and *E* showed no digestive activity in four days. Indeed, they were more stable than the control (F.) of 48 hours "lytic time," suggesting the presence of an *anti*-tryptase factor. In *D*, fibrinolysis was complete in 20 hours, with *C* not far behind (29 hours). *B* (one-hour sample) was weakly postive, with digestion about half completed in 36 hours, and quite clear in 40 hours (when the control was just beginning to lyse). All tests indicate very little enzyme and fall below the satisfactory working range of the method. Even so, the data are valuable and, allowing for the plasma dilution (final = 1:25), it can be stated with some assurance, that the rabbit plasma (serum) materials tested have a trypsin (N.T.) equivalence (by the fibrinolytic test) of less than 1:40,000. This is equivalent to .025 mg. N.T., or 1.06×10^{-4} $[T.U.]_{\mathrm{m}}$, per c.c. Comparison with dog, human, and other plasmas will be deferred for a subsequent communication, but it may be stated that the *rabbit* data are significantly lower than in carnivorous species. This offers a rational explanation of the exceptional stability and enzyme-free character of Parfentjev's "rabbit clotting globulin," which makes it so excellent a thrombin for the assay test and other purposes.

Owing to the relative paucity of serum-tryptase, rabbit blood behaves somewhat differently from dog blood, in clotting experiments, under conditions similar to those employed by Tagnon.[15]

COAGULATION TESTS

Room temperature = 23° ± 2° C. Clotting-times for 1.0 c.c. weak fibrinogen solution *plus* 0.5 c.c. chloroform-treated materials. Character of clot (in parenthesis, Table II): ++ = complete (tube invertible); + = incomplete. The significance of the experimental data may be interpreted as follows:

1. When plasma is shaken with $CHCl_3$ and the tryspin-inhibitors thereby "neutralized," small amounts of serum-tryptase are activated. By its *thromboplastic action*,[16, 4] notwithstanding depression of the Ca-ions due to the citrate present, the enzyme activates a part of the prothrombin to thrombin. This increases to a low "peak" level and then decreases. We do not wish, at this time, to discuss the several possibilities which might account for the later decrease, but adsorption on fibrin, "immediate" anti-thrombins (i.e. "metathrombin" formation) and progressive thrombinolysis may be mentioned. The persistence of such a weak thrombin at all shows that tryptic thrombinolysis is negligible. Considerable unaltered prothrombin persists in the $CHCl_3$-treated plasma, as shown by (5-minute) recalcification of a sample and adding to fibrinogen, when solid clotting occurs in eight minutes. The recalcification produces no clot in the $CHCl_3$-plasma itself; neither does added thrombin, thus showing that the fibrinogen has all disappeared. It is, in fact, deposited as a fibrin coagulum (flocculant) adjacent to the chloroform layer in bottle A. No such appearance is seen in B — E. The thrombin formed by the tryptase action is sufficient to account for the fibrin deposit. The clots in the tests with

fibrinogen, although incomplete, were perfectly stable for three days. The important conclusion is that serum tryptase, in amounts too small to be detected by any of its other actions (viz., lysis of fibrin or thrombin, or their precursors) is, nevertheless, able to exert the typical thromboplastic action in the conversion of prothrombin to thrombin.

2. In recalcified plasma, thrombin production proceeds in the *natural* manner but is doubtless aided by the thromboplastic action of small quantities of tryptase liberated by the $CHCl_3$-treatment. This might not be true if the conversion of prothrombin to thrombin were completed prior to the addition of chloroform. In the present experiment, however, the conversion was definitely retarded and for an unusual reason, namely, too great an excess of Ca, occasioned by the inadvertent use of stock (N/2) instead of the customary (N/10) $CaCl_2$. Thus the preliminary plasma clotting required twenty minutes, instead of three minutes noted on repetition with N/10 $CaCl_2$. Thinking that the experiment might be instructive, the first sample was chosen for the $CHCl_3$ treatment and it did yield the pertinent observation that additional thrombin continued to form during the twenty-four hours of contact of the serum with chloroform. No evidence of tryptic thrombinolysis was obtained. The fibrinolytic test proves the paucity of tryptase, but the assay is definitely positive and in the clotting-test there was enough enzyme to produce clot retraction in three and one-half hours and the clot had almost lysed by the end of the third day, when observation was discontinued.

3. The addition of enzyme-free thrombin to citrated plasma produces complete conversion of fibrinogen to fibrin and unequivocal liberation of tryptase (see lytic tests). There is no evidence of any *immediate* thrombin formation from the plasma prothrombin. The slight excess of added thrombin is sufficient to account for the weak (60-minute) clot after the one-hour $CHCl_3$ treatment. By the end of six hours no thrombic activity is left, probably owing to destruction by the tryptase formed. After twenty-four hours a (weak) thrombic action reappears. This is attributed to the slow thromboplastic action of the enzyme on the prothrombin of the serum. That an abundance of prothrombin persists unaltered in the serum is clearly shown by a test in which a (30-minute) recalcified sample of the twenty-four-hour serum clots a test fibrinogen solidly in eighty *seconds*. The lytic tests reveal the small quantity of tryptase responsible for these effects. Retraction of the weak original clots, (*A*) and (*C*), occurred on the second day but lysis was far from complete by the end of the third day.

4. That well-washed fibrin contains traces of thrombin, which can be eluted with saline, is the basis for the old Gamgee-Howell method of thrombin preparation. The slight increase in the thrombin obtained after six hours is probably due solely to the longer period of extraction. Enzymic thrombinolysis may account for the falling off in thrombic activity in the twenty-four-hour sample. The persistence of such a weak thrombin in the presence of the serum tryptase revealed by the fibrinolytic tests is remarkable, however, and suggests that adsorption onto the fibrin protects the thrombin (to some extent) from the digestive action of small quantities of enzyme, which are obviously also adsorbed onto the fibrin clot and eluted with saline. The positive tests of *C* and

D show that the tryptase liberated by the $CHCl_3$ treatment is divided between the serum and the clot. Tagnon[15] was, therefore, well advised in shaking the *whole* clotting mixture with $CHCl_3$ in his fibrinolytic experiments and we propose to continue this practice in the clinical examination for human blood tryptase.

5. The serum experiments show a trace of persistent thrombin, which is to be expected in the absence of demonstrable tryptase activity.[6] The fibrin in the clotting tests was still solid after three days.

The close parallelism between tryptic and clotting activities, evident in these experiments, suggests an autocatalytic process of enzyme activation.

DISCUSSION

We should like to point out the undoubted confirmation, which Tagnon's[15] data afford, of the views expressed in frequent communications from the Michigan laboratories during the past three years. We have delayed full consideration of the role of serum tryptase in fibrinolysis because of a conviction that this is a minor and variable side-action of the role of the enzyme factor in blood-clotting. The present experiments with rabbit blood add emphasis to this view.

In evaluating the experiments of Eagle and Harris,[16] Tagnon is correct in pointing out that they compared crystalline trypsin to Ca + "thromboplastin" without attempting a definition of the latter term. Eagle's work must be credited with stimulating renewed interest in proteolytic enzymes and their relationships to blood clotting, although considerable older data appear in the literature and we must give a niche to the long-forgotten study of Collingwood and MacMahon, 1913,[17] suggesting a relationship of trypsin to progressive antithrombic action.

In extending Eagle's observations, Ferguson (op. cit.) has sought to demonstrate that the *natural* serum tryptase (long known to enzyme chemists) actually *is* the chief thromboplastic factor of the blood plasma. While it can act under conditions of not-too-great depression of the Ca-ionization as shown by Eagle,[16] Ferguson[3, 4] points out that Ca and cephalin are essential for optimal prothrombin activation. Particularly noted[3] in this connection is the "stability" of the clots obtained, which is a phenomenon again emphasized by Tagnon[15] in his rather obscurely presented view concerning "inhibitory" actions of "thromboplastin" (and "prothrombin"). Current studies of trypsin *inhibitors* are yielding very interesting data, which will appear from the Michigan laboratories in due course. That a deficiency of serum tryptase is the "bane of the Hapsburgs" is the Ferguson (1939)[9] theory of the causation of *hemophilia.*

SUMMARY

A new fibrinolytic method of protease (especially tryptase) assay is detailed. It can detect small differences within a range of (final) concentrations of 1:40,000 to 1:1,000,000 crystalline trypsin (1 mg. X. T. = 4.24×10^{-3} [T.U.] m.). While the wide fields of usefulness of the new methods are illustrated only at the extreme limit by the rabbit blood clotting experiments here pre-

sented, it is significant that this animal yields serum tryptase not in excess of 1:40,000 (N.T.) per cubic centimeter of materials tested and that appreciable quantities of enzyme liberation require coagulation(!) whether by added thrombin or by recalcification. With these relatively small amounts of enzyme (probably a species peculiarity), "thromboplastic" conversion of prothrombin to thrombin yields stable thrombin and stable fibrin clots.

REFERENCES

1. Ferguson, J. H.: (a) A New Blood-Clotting Theory, Science 97: 319, 1943. (b) The Role of Blood Clotting Anomalies in the Hemorrhagic Diseases, J. Lab. & Clin. Med. 26: 52, 1940.
2. Oppenheimer, C.: Die Fermente und ihre Wirkungen. Vol. 2, ed. 5, Leipzig, 1926, G. Thieme.
3. Ferguson, J. H., and Erickson, B. N.: The Coagulant Action of Crystalline Trypsin, Cephalin, and Lung Extracts, Am. J. Physiol. 126: 661, 1939.
4. Ferguson, J. H., and Erickson, B. N.: Calcium and Cephalin in Relation to the Clotting Power of Crystalline Trypsin, Proc. Soc. Exper. Biol. & Med. 40: 625, 1939.
5. Ferguson, J. H.: Stability of Prothrombin in the Presence of Thrombin, Proc. Soc. Exper. Biol. & Med. 46: 80, 1941.
6. Glazko, A. J., and Ferguson, J. H.: Kinetics of Thrombin Inactivation as Influenced by Physical Conditions, Trypsin, and Serum, J. Gen. Physiol. 24: 169, 1940.
7. Ferguson, J. H.: The Action of Heparin, Serum Albumin (Crystalline), and Salmine on Blood-Clotting Mechanisms (in Vitro), Am. J. Physiol. 130: 759, 1940.
8. Ferguson, J. H.: The Flocculation Maximum (pH) of Fibrinogen and Some Other Blood-Clotting Reagents. (Relative Turbidimetry With the Evelyn Photoelectric Colorimeter), J. Gen. Physiol. 25: 607, 1942.
9. Ferguson, J. H.: The Clotting of Hemophilic Plasma by Thromboplastic Enzyme, Am. J. Physiol. 126: 669, 1939.
10. Feissly, R.: Nouvelles etudes sur l'hemophilie. I. Separation des facteurs plasmatiques intervenant dans la formation de la thrombine, Helvet. med. acta 8: 823, 1941.
11. Ferguson, J. H.: Estimation of Trypsin by Fibrinolysis (Preliminary Communication), Proc. Soc. Exper. Biol. & Med. 51: 73, 1942.
12. Parfentjev, I. A.: A Globulin-Fraction in Rabbit's Plasma Possessing a Strong Clotting Property, Am. J. M. Sc. 202: 578, 1941.
13. Anson, M. L., and Mirsky, A. E.: The Estimation of Trypsin With Hemoglobin, J. Gen. Physiol. 17: 151, 1933.
14. Christensen, L. R.: Quantitative Determination of Activity of Streptococcal Fibrinolysin, Proc. Soc. Exper. Biol. & Med. 46: 674, 1941.
15. Tagnon, H. J.: The Significance of Fibrinolysis in Mechanism of Coagulation of Blood, J. Lab. & Clin. Med. 27: 1119, 1942.
16. Eagle, H., and Harris, T. N.: The Coagulation of Blood by Proteolytic Enzymes, J. Gen. Physiol. 20: 543, 1937.
17. Collingwood, B. J., and MacMahon, M. T.: (a) The Anti-Coagulants in Blood and Serum, J. Physiol. 45: 119, 1912. (b) The Nature of Thrombin and Anti-Thrombin, J. Physiol. 47: 44, 1913.

EFFECT OF TOURNIQUETS ON VENOUS BLOOD SUGAR VALUES*

Winifred C. Loughlin, M.D., Herman O. Mosenthal, M.D., and
Ruth Halpern, B.A., New York, N. Y.

THE influence on the blood sugar level of a tourniquet employed to distend the veins in carrying out venepuncture has never been considered, and yet obvious possibilities exist.

METHODS

The present determinations were carried out, using a blood pressure cuff with mercury manometer attached as a tourniquet. A control series of observations was made without the use of a tourniquet. The tension applied to the upper arm was either above the systolic arterial pressure, "systolic tourniquet," or at or above the diastolic pressure, but distinctly below the systolic pressure, "diastolic tourniquet." Each one of the three procedures was carried out on five normal subjects in the fasting state. An interval of several days elapsed between each of the three experiments. The blood sugar determinations were carried out over a period of about six minutes.

The venous blood sugar estimations were made according to the method of Folin and Malmros[1] as modified for the photoelectric colorimeter by Saifer, Valenstein, and Hughes.[2] The determinations were carried out in duplicate on a carefully standardized photoelectric colorimeter.

TABLE I

CONTROL LEVELS OF BLOOD SUGAR MG. PER 100 C.C., CORRESPONDING TO CHART

	CASE 1	CASE 2	CASE 3	CASE 4	CASE 5
No Tourniquet	95	108	100	111	104
Arterial Tourniquet	88	98	84	102	89
Venous Tourniquet	81	93	99	125	103

RESULTS

The blood sugar series determined without a tourniquet serve as a control for the subsequent ones. The variations from the initial blood sugar in the 28 blood sugar estimations comprised in this group. varied above 6 mg. per 100 c.c. in only two instances: Case 2, 10 mg. below the control level, and Case 4, 10 mg. above the control level. From these results it may be concluded that changes of 10 mg. or less are within the limits of error for the present procedure.

The curves obtained when an arterial tourniquet was applied are distinctly more variable than when no tourniquet was used. In only three blood sugar estimations, however, did the changes exceed the 10 mg. limit set as the limit of error: 15 mg. per 100 c.c. deviation from the control in Case 2, and two values

*Sponsored by the New York Diabetes Association.
Received for publication, November 13, 1942.

of 11 mg. in Case 3. It would seem justifiable to conclude that the arterial tourniquet effects no very significant changes in the venous blood sugar; the greater variability of the blood sugar with the arterial tourniquet, as compared with the venous blood sugar values when no tourniquet was applied, may be ascribed to the time required to inflate the cuff, when for a few seconds the venous tourniquet would be active and induce such variations as are discussed in the next paragraph.

The results with the venous tourniquet are distinctly different. They show changes from the control blood sugar varying from 18 to 24 mg. per 100 c.c. In no case was the variation less than 18 mg. per 100 c.c.; in four instances the blood sugar rose; in one the principal change was a drop; the most consistent fluctuation was a rise in the blood sugar followed by a drop.

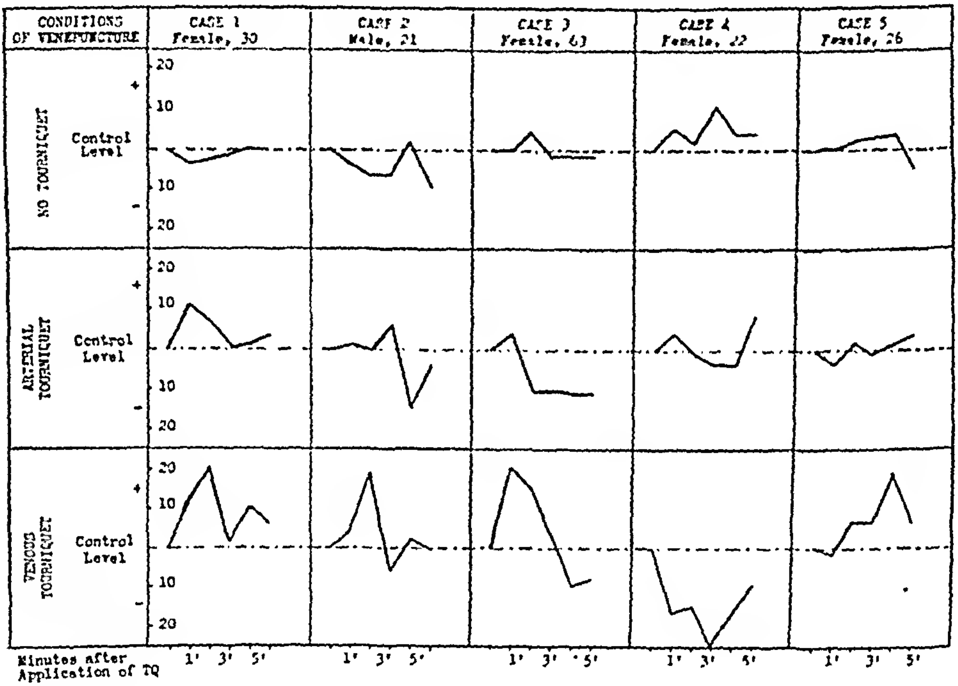


CHART I.—VARIATIONS OF THE VENOUS BLOOD SUGAR FROM THE CONTROL IN FASTING NORMAL SUBJECTS WITHOUT A TOURNIQUET, WITH AN ARTERIAL TOURNIQUET AND A VENOUS TOURNIQUET. RISE IN BLOOD SUGAR IS INDICATED BY THE HEIGHT OF THE CURVE ABOVE THE CONTROL LEVEL LINE, A DROP BY THE DEPTH OF THE CURVE BELOW THE CONTROL LEVEL LINE. THE CONTROL BLOOD SUGARS VARIED FROM 81 TO 125 MG. PER 100 C.C. SEE TABLE I.

DISCUSSION

Without a tourniquet the venous blood sugar level, obtained at minute intervals over a six-minute period, shows no variations that cannot be ascribed to the limit of error inherent in the procedure employed. The results after the application of a venous tourniquet indicate that some adjustments occur that will cause the blood sugar to undergo fluctuations that are significant from the clinical point of view. Such changes deviate about 20 mg. per 100 c.c. from the expected value; they generally take place within two minutes after the application of the venous tourniquet. As a rule there is a rise of the blood sugar,

though in one instance (Case 4) there was a marked fall. These initial variations in the venous blood sugar are transient, and are followed by a shift in the opposite direction after the tourniquet has been in effect two or three minutes.

The increase of the glucose concentration in the venous blood while a venous tourniquet is acting may be ascribed to the forcing of arterial blood with its higher sugar content into the occluded veins.

Ebert and Stead[3] have shown that one of the effects of venous tourniquets on the extremities is a reduction of the plasma volume in the blood vessels, resulting from a transudation of fluid into the surrounding tissues. Since the concentration of glucose is greater in the plasma than in whole blood, such a diminution of plasma volume would account for a lowering of the blood sugar when the amount of plasma was less and the red blood cell content was greater.

These two factors, increased accumulation of arterial blood in the veins and diminished plasma volume, occurring at different intervals after the application of a venous tourniquet, would account for either a rise or a drop in the venous blood sugar. The absence of these two influences with no tourniquet at all would explain the stabilization of the venous blood sugar under those circumstances.

Venous blood sugars determined after the application of an arterial tourniquet are slightly more variable than those when no tourniquet is used. This may be accounted for by the transient phase of venous tourniquet pressure in applying the arterial tourniquet. It is probable that if the tourniquet would be adjusted with great speed, the venous blood sugar figures derived from the use of the arterial tourniquet would duplicate those obtained without a tourniquet.

CONCLUSIONS

Without a tourniquet, the venous blood sugar levels remain constant over a period of five minutes. The application of a venous tourniquet results immediately in significant changes in the venous blood sugar amounting to 20 or even 25 mg. per 100 c.c. as either an increase or a diminution from the control value. The use of an arterial tourniquet causes some variations in the venous blood sugar, which, however, are much less marked than those obtained with the venous tourniquet. For the greatest accuracy a tourniquet should not be used in obtaining blood for venous blood sugar determinations.

REFERENCES

1. Folin, O., and Malmros, H.: An Improved Form of Folin's Micro Method for Blood Sugar Determinations, J. Biol. Chem. 83: 115, 1929.
2. Saifer, A., Valenstein, F., and Hughes, J. P.: Photometric Determinations of Sugar in Biological Fluids by Ferricyanide Reduction, J. Lab. & Clin. Med. 26: 1969, 1941.
3. Ebert, R. V., and Stead, E. A., Jr.: The Effect of the Application of Tourniquets on the Hemodynamics of the Circulation, J. Clin. Investigation 19: 561, 1940.

AN APPARATUS FOR STORAGE OF GAS-FREE SOLUTIONS FOR USE WITH THE VAN SLYKE GASOMETRIC METHODS*

R. H. GOETZ,† M.D., CAPE TOWN, SOUTH AFRICA

VARIOUS apparatus have been designed to keep the reagents used for gasometric analysis gas-free for some length of time. Most of them, however, as pointed out by Guest and Holmes,[1] have the disadvantage that the alkaline solutions have to pass a glass stopcock,[2, 3] the grease of which does not

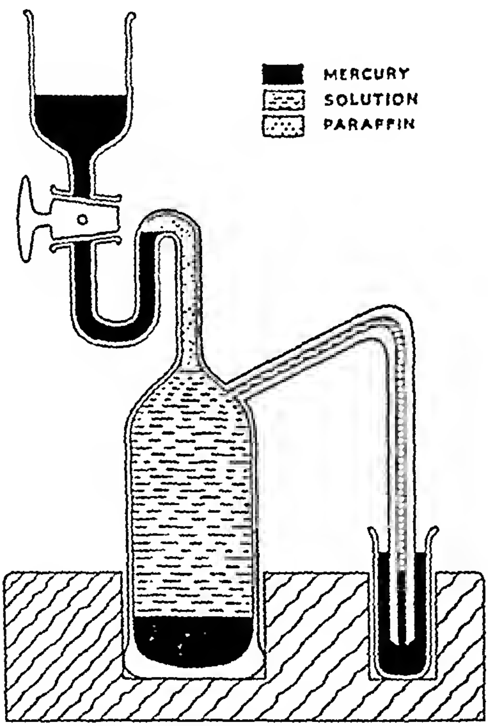


Fig. 1.

hold long against the alkaline solutions and leakages occur easily. The apparatus of Holmes[4] eliminates this difficulty, but it has the disadvantage of having to be made by an expert glass blower.

The following apparatus has been designed to take the place of more complicated pipettes; it has at the same time the advantage that only mercury or paraffin comes into contact with the stopcock.

*From the Department of Surgery, University of Cape Town.
†Jacobus Stephanus Marais Research Assistant.
The apparatus was blown for us by Mr. V. J. Hinkley.
I am indebted to the Government Grant Committee for a grant covering the expenses.
Received for publication, November 18, 1942.

As seen in Fig. 1, the vessel consists mainly of four parts: (1) the upper storage chamber for the mercury, (2) the lower storage chamber for the solution, (3) an S-shaped tube connecting both vessels and provided with a stopcock, and (4) a delivery tube which is immersed into a small test tube filled with mercury, providing the seal.

Filling of the Apparatus.—Before filling the vessel with the solution, it is filled with liquid paraffin, in which process special care has to be taken that the paraffin does not contain any air bubbles. Following this, the paraffin is displaced by the solution in the manner indicated in Fig. 2. The delivery tube is fitted into the bottom of the cup of the Van Slyke, a small rubber ring making

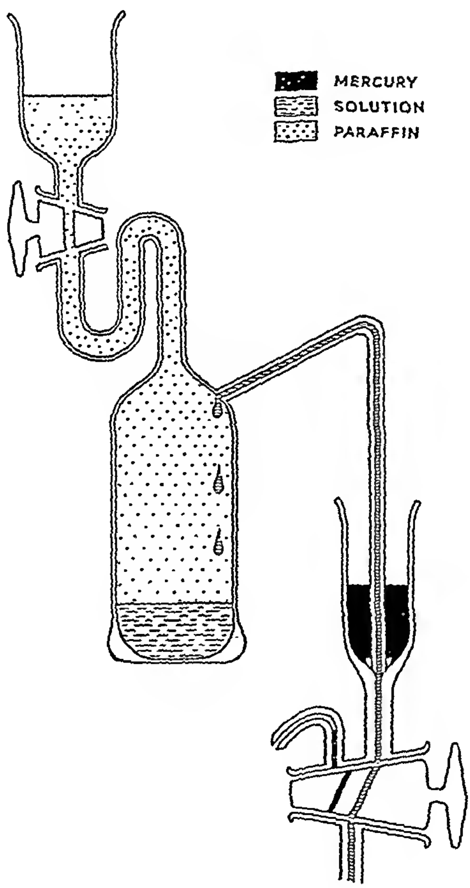


Fig. 2.

the connection airtight, and mercury sealing the connection as well. With the stopcock of our vessel open, and the leveling bulb of the Van Slyke in the upper ring, the upper cock of the Van Slyke pipette is opened and the gas-free solution is forced slowly up the delivery tube of our vessel. It then drops through the paraffin to the bottom of the storage chamber, displacing the liquid paraffin into the upper chamber of the vessel. The solution is thus collected in the storage chamber without coming into contact with the air. When all the solution has been delivered to the storage vessel, the stopcock of the vessel is closed, and the paraffin in the upper vessel is recovered. About 10 c.c. of mercury are

then poured into the upper chamber; part of it is allowed to fill the S-shaped connection, providing a seal. The vessel may then be placed in a convenient stand, its delivery tube being immersed into mercury as shown in Fig. 1. This provides a complete seal, and solutions have been stored gas-free in this way by us for many months.

The Use of the Apparatus.—In order to get the solution into the Van Slyke during an analysis, the upper storage chamber is filled with sufficient mercury. On opening of the stopcock, the mercury by its own gravity will flow from the upper into the lower storage chamber, displacing the solution via the delivery tube. After closing of the stopcock and after returning of the vessel into the stand, complete sealing is again obtained. Mercury has to be added from time to time in the upper vessel, as it is used for replacing the solution.

The dimensions of the apparatus can be clearly seen from the accompanying figures. Actually, any size for the vessel storing the solution is possible, but for practical purposes 100 c.c. capacity for the chamber holding the solution was found most suitable. The upper (mercury) chamber should conveniently hold about 30 c.c. in order to take the paraffin which is being displaced when filling, since 25 c.c. of solution may be made air-free at one time with the Van Slyke. The delivery tube is made of capillary tubing having a bore of 1.5 mm. It is constructed in such a way that the vertical part of it during filling and delivery of the solution is sufficiently far away from the cup of the Van Slyke as to allow smooth operation of the latter's stopcock.

SUMMARY

A self-sealing apparatus for the storage of gas-free solutions is described, which has the following advantages:

1. It eliminates the use of rubber parts and of glass stopcocks where the grease could be dissolved by the alkaline solution stored in the vessel.

2. It can be easily filled.

3. It provides a mercury seal for the delivery tube.

4. The apparatus is characterized by its simplicity and therefore can be easily made by a moderately experienced glass blower. It is much less liable to break than the more complicated vessels in use.

The vessel has been found most convenient, especially under such conditions where gasometric analyses are undertaken only at long intervals.

REFERENCES

1. Guest, J. M., and Holmes, F. E.: A Pipette for Storage of Air-free Reagents Used in Gasometric Analysis, J. Biol. Chem. 110: 781, 1935.
2. Van Slyke, D. D., Page, I. H., and Kirk, E.: A Manometric Micromethod for Determination of Carbon in Organic Compounds, J. Biol. Chem. 102: 635, 1933.
3. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry—Methods, Baltimore, 1932, Williams & Wilkins Co., pp. 109, 363.
4. Holmes, F. E.: Self-Sealing Vessels for Storage of Solutions Used in the Van Slyke Gasometric Methods, J. Biol. Chem. 113: 411, 1936.

A RAPID MICROANALYTICAL METHOD FOR LEAD IN URINE*

SIDNEY KAYE,† SAN JUAN, PUERTO RICO

A METHOD is herein described for the rapid microanalysis of lead in urine. Aub and associates[1] mention the fact that ammonium hydroxide quantitatively precipitates lead as lead phosphate from urine. If such be the case, this would afford an excellent means of separating the lead from interfering organic matter in urine. Experimentation,[2] however, showed that excess ammonia did not result in a 100 per cent recovery. Fairhall[3] states that there is a 100 per cent precipitation of lead with ammonia at pH 10. This was found to be experimentally correct.

Several excellent procedures have been described for the destruction of organic matter in urine in preparation of the sample for the determination of lead. These employ either the dry muffle heat below 500° C. or the various acid oxidation modifications. Although fairly rapid, these methods are not as rapid as the phosphate precipitation. Moreover, in the wet oxidation methods, the quantities of urine analyzed have been so reduced that slight deviations become appreciable and significant when calculated on a liter or per diem basis. Larger samples of urine may, of course, be digested, but then there is time consumption in concentrating the volume.

PRINCIPLE OF METHOD

The method is based upon the total precipitation of lead by the addition of ammonium hydroxide to urine. Lead phosphate is very insoluble and is easily filtered out along with the alkaline earth phosphates of calcium and magnesium. This precipitate is subsequently dissolved with hot dilute nitric acid and is heated for a short time to oxidize any adhering organic material. Citric acid is added to keep the phosphates in solution during later neutralization, and potassium cyanide is added to form complex ions with interfering metals.** When the pH is adjusted between 9 and 10, the lead is extracted with dithizone†† to separate it from extraneous salts and interfering substances. Once the lead is separated, it is then split from the dithizone and titrated with standardized dithizone for an accurate determination. Under the above conditions only bismuth, tin, thallium, and lead react with dithizone. To remove the interference of bismuth and tin, these are previously extracted with dithizone while the solution is yet acidic. Lead is not extracted in this acid solution. Thallium, as a source of interference, has not been removed, but its occurrence is rare and unlikely.

---

*From the Puerto Rican Department Laboratory, San Juan, P. R. Colonel V. H. Cornell, M.C., Commanding.

**Cd, Zn, Cu, Co, Ni.

†Captain, Sanitary Corps, Chemist and Toxicologist, Army of the United States.

††Dithizone is the abbreviated name for diphenylthiocarbazone.

Received for publication, Dec. 3, 1942.

REAGENTS

All solutions should be kept in pyrex glass bottles. Water is distilled from an all-pyrex still. Ammonia water and nitric acid should have low lead content.

Potassium Cyanide Solution: 10 Gm. of potassium cyanide (low in lead) dissolved in water to make 100 ml.

Citric Acid Solution: 200 Gm. of citric acid (low in lead) dissolved in water to make 500 ml.

Standard Lead Solution: 160 mg. of lead nitrate dissolved in 1,000 ml. of water. One milliliter is equivalent to 100 gamma* of lead.

Dithizone 1 for Preliminary Extraction: 25 mg. of dithizone dissolved in 400 c.c. of chloroform.

Dithizone 2 for Titration: 100 ml. of dithizone 1 diluted with 100 ml. of chloroform. This is standardized against known lead. Approximate value is 1 c.c. = 5 gamma lead. If kept in a brown bottle and in a cool place, the titer will not change for several months. This titer is satisfactory for amounts of lead near normal values. However, if larger amounts of lead are expected (this is determined approximately by the amount of dithizone required for the preliminary extraction), a stronger titer may be prepared. Dithizone 1, when standardized, is approximately 1 c.c. = 10 gamma lead. The titer should be calculated to at least one decimal unit.

All glassware should be of pyrex quality.

METHOD IN DETAIL

Description.—The total twenty-four-hour specimen of urine is collected and the volume measured. One-third of this volume is taken for analysis. A few drops of phenolphthalein and then dropwise ammonium hydroxide are added until the urine turns pink (pH 9 to 10). This is well shaken and stoppered and the precipitate which forms is allowed to settle for about thirty minutes. The precipitate is then separated by being filtered through a Buchner funnel with suction and refiltered if necessary. The precipitate is washed with several small portions of alcohol. The filter paper is then inverted so that the precipitate now faces the perforations of the funnel. Approximately 30 to 50 ml. of hot 3 per cent nitric acid are successively poured over the precipitate until it either dissolves or is completely transferred with the filtrate to the flask. This is more easily accomplished without suction. The total redissolved precipitate is then gently boiled for approximately twenty minutes. It is adjusted to a pH of from 3 to 3.5 (lower range of methyl orange) with the dropwise addition

*Gamma = 0.001 mg.

of ammonium hydroxide and transferred to a Squibb separatory funnel. It is then shaken out with 5 to 10 ml. of dithizone 1, and the dithizone-chloroform layer is discarded. This extracts and eliminates the possibility of either tin or bismuth appearing later as an interference. Lead is not extracted at this pH. Then 15 ml. of citric acid are added and the solution neutralized with ammonium hydroxide. With the addition of 2 ml. of potassium cyanide, the solution is ready for the lead extraction. In an ammoniacal solution and in the presence of potassium cyanide, dithizone has a selective affinity for lead, forming a cherry red complex of lead dithizone which is soluble in chloroform.

A small quantity of dithizone is run into the solution from a burette and vigorously shaken. The rapid appearance of a cherry red chloroform layer indicates the presence of lead. After each addition of dithizone, vigorous shaking is necessary and the colored chloroform layer is separated carefully and transferred to another separatory funnel. The last traces of lead dithizone are washed through with several small portions of chloroform. These are also collected and saved. The proximity of the end point (complete removal of lead) may be roughly estimated by the diminution of intensity of the cherry red color. If it is very red, a larger amount of dithizone may be added and again shaken and separated. After each shaking, the funnel is inverted and the stopcock opened to relieve the pressure. This is continued until all the lead has been extracted, which is indicated when further addition of dithizone does not impart a cherry red color to the chloroform layer, but on vigorous shaking a green color persists due to excess dithizone. The presence of many extraneous salts prevent a sharp end point (cherry red to green). Therefore, the first titration-extraction is not a reliable indication of the quantity of lead present. The end point is difficult to see because of the lag of the transition of colors (cherry red, pink, violet, blue, green). To rectify this, the several portions of extracted lead dithizone should be carefully collected and saved. These combined chloroform extracts are shaken with 15 ml. of 1 per cent nitric acid which quantitatively separates the lead into the aqueous solution. The green chloroform layer is discarded. The aqueous layer is washed by shaking with a fresh portion of chloroform to remove any residual dithizone. To the solution are added several drops of phenolphthalein and dropwise ammonia until alkaline, then 2 ml. of potassium cyanide, and lastly dropwise citric acid until the solution is just faintly pink. If the end point is overrun, a drop of dilute ammonium hydroxide will adjust it.

The solution is now titrated with standard dithizone 2. The dithizone may be added in one large portion to within 5 ml. of the total amount required in the first extraction (when cherry red color started to change). The separatory funnel is thoroughly shaken, allowed to stand until the layers separate, and the red chloroform layer is then removed. From this point, the titration continues carefully. The dithizone is delivered in successive small quantities (approximately 0.5 ml. portions), and it is necessary before each shaking to add approximately 1 ml. of chloroform so as to have enough volume to see the color. After each addition, the solution should be vigorously shaken, allowed to settle, and then the chloroform layer run out and discarded. This is continued until the end point is reached, which is a persisting light green color in the chloroform layer.

For standardization a known lead solution is titrated with the dithizone solution using the procedure previously described. Since the lead is in pure solution, the preliminary titration-extraction is not necessary. The quantity of lead in the standard used, divided by the number of milliliters of dithizone solution required, represents the dithizone lead equivalence.

A lead blank is determined for the reagents. However, since the reagents should be of highest quality or are previously deleaded, the blanks should be relatively low.

TABLE I

ANALYSIS OF 500 ML. PORTIONS OF POOLED URINE

LEAD ADDED (GAMMA)	TOTAL LEAD RECOVERED (GAMMA)	EVALUATION OF THE AMOUNT OF LEAD ADDED (GAMMA)
00	23	--
00	22	--
25	47	25
25	47	25
50	73	51
50	70	48
100	118	96
100	123	101
150	169	147
150	172	150
200	212	190
200	224	202
500	505	483

Table I indicates that this method for lead is applicable to urine, using dithizone as a standard solution. The maximum error was ±5 per cent.

SUMMARY

1. Excess ammonium hydroxide as prescribed by Aub and associates for the precipitation and absorption of lead is unsatisfactory.

2. The lead may be totally separated from urine at pH 10 as suggested by Fairhall.

3. The lead is quantitatively determined by an extraction-titration procedure with dithizone reagent.

4. This method for determining lead in urine is rapid, sensitive, and reliable, and the maximal deviation is ± 5 per cent.

REFERENCES

1. Aub, Fairhall, Minot, and Resnikoff: Lead Poisoning, Baltimore, 1926, Williams & Wilkins Co.
2. Kaye, S.: A Study of the Analytical Methods for the Detection of Lead From Biological Materials. Master of Science Thesis, New York University, 1939.
3. Fairhall, L. T., and Keenan, R. G.: A Rapid Method for the Microanalysis of Lead, J. Am. Chem. Soc. 63: 3076, 1941.
4. Willoughby, C. E., Wilkins, E. S., and Kraemer, E. O.: Determination of Lead, Industrial and Engineering Chemistry, Analytical Edition 7: 285, 1935.

The Journal of Laboratory and Clinical Medicine

VOL. 28 | JULY, 1943 | NO. 10

PROGRESS

THE VERIFICATION TEST IN THE SEROLOGY OF SYPHILIS*

REUBEN L. KAHN, D.SC., ANN ARBOR, MICH.

INTRODUCTORY

1. *The Verification Test—The Search for Characteristics Which Differentiate Specific From Nonspecific Serologic Reactions.*—Studies in progress in this laboratory aimed at the detection of differential characteristics of true and false positive serologic reactions have thus far led to two observations which have afforded a basis for several practical procedures. The observations are that, within certain limits, (1) specific serologic reactions tend to become stronger and nonspecific reactions weaker as the reacting temperature is increased; and (2) specific serologic reactions tend to become stronger and nonspecific reactions weaker as the salt concentration of the reacting system is increased. The practical procedures are (1) differential temperature technique, (2) triple quantitative technique, and (3) salt dispersibility technique.

2. *Occurrence of Biologic False Positives.*—The nature of biologic false positive reactions is still obscure. It is believed that the following two factors largely determine the occurrence of these reactions with serodiagnostic tests: (1) a tendency to give such reactions on the part of certain individuals, and this tendency may be constitutional in nature; and (2) some pathologic disturbance (fever, cancer, etc.) or biologic imbalance (pregnancy, vaccination, etc.) serving as the immediate cause of such reactions, although the reactions are encountered also in individuals apparently free from pathologic disturbance or biologic im-

*From the Clinical Laboratories, University of Michigan Hospital. Aided by a grant from the United States Public Health Service.

This article is not a review on false positives and their detection. The article considers only the present status of the verification test. The experimental studies upon which this article is based were carried on in this laboratory in association with Elizabeth B. McDermott, Jacob Adler, Stanley Marcus and other members of the staff, and will be published elsewhere.

balance. The occurrence of positive reactions in the absence of syphilis is unique *in that many individuals who show no tendency to give these reactions* with serodiagnostic tests begin to give them with the increase in the sensitivity of the tests, indicating that seronegative persons possess latent potentialities for manifesting such reactions.

3. *Relation Between Biologic False Positives and Serologic Reactions Given by Lower Animals.*—Studies carried out in this laboratory indicate that false positive reactions given by human beings and the positive serologic reactions given by pigs, horses, rabbits, and other animals possess similar characteristics. Thus, these two types of reactions are most marked (1) when the tests are performed at cold temperature (1° C.), and (2) when the salt concentration of the reacting system is reduced to a minimum. These findings suggest that a close relationship exists between human and animal reactions of nonsyphilitic origin given by serodiagnostic tests. In this laboratory, indeed, animal sera are commonly used for studies of false positive reactions.

4. *Basis of Biologic False Positives.*—Tissue extract antigens of excessive sensitivity can be prepared which will give about 40 per cent of positive serologic reactions in nonsyphilitic persons. If the serologic tests with excessively sensitive antigens are performed at cold temperature, the nonspecific sensitivity can be raised to about 80 per cent. If the tests with excessively sensitive antigens are performed at cold temperature with unheated sera (instead of with sera heated for 30 minutes at 56° C.), the nonspecific sensitivity reaches about 98 per cent. Certain lower animals (horses, pigs, chickens) also give 100 per cent of positive reactions when the tests are performed under the same conditions. These findings suggest that there exists a "universal" serologic (nonsyphilitic) reaction, given by human beings and certain lower animals, which is best manifested under conditions of low temperature. It was shown before that the biologic false positive reaction given by serodiagnostic tests is similarly best manifested under conditions of low temperature. Hence, it would seem reasonable to assume that the two reactions are related to one another and that the universal reaction may be the basis of biologic false positives.

5. *Biologic False Positives in the Presence of Syphilis.*—Biologic false positives may be given by persons with syphilis. Thus, a certain person has a tendency to give false positives, let us say, when he contracts a cold. In due time, he becomes infected with syphilis and he begins to give specific reactions. Then, when he contracts a cold, he is likely to give at the same time both specific and nonspecific reactions. Should he become specifically seronegative following therapy, he will give only the nonspecific reaction when from time to time he has a cold. Also, certain neurosyphilitic patients treated with malaria give specific serologic reactions before malarial therapy, both specific and nonspecific reactions soon after the therapy, and in due time only specific serologic reactions again.

6. *Technical and Biologic False Positives.*—False positives are generally classified as either technical or biologic, but it is not always possible to draw a line of demarcation between them. A false positive given by a supersensitive test, for example, may be interpreted by some as technical, because the antigen

in this test shows a tendency to give, let us say, 5 per cent of false positive reactions. But the fact that 5 particular persons in 100 gave these reactions would indicate that the sera in these persons differed biologically (biochemically or physiochemically) from the sera in the remaining 95 persons, thereby rendering these reactions biologic false positives. Experience indicates that any serodiagnostic reaction suspected of being a false positive, whether biologic or technical, may be investigated with the verification test.

7. *Verification Results and Clinical Findings.*—The dependability of laboratory results in a given infection is generally determined by the extent to which these results are corroborated by clinical findings of that infection. Unfortunately, verification reports of false positives cannot be corroborated by a definite clinical indicator. Negative clinical findings of syphilis represent *the* clinical indicator of false positives, and negative findings cannot have the same value as positive findings. Yet, in the diagnosis of false positives, the establishment of negative clinical findings of syphilis is obviously of first importance. When such findings corroborate verification results of false positives, the significance of these results immediately becomes fortified, just as the significance of positive Widal reactions becomes fortified by clinical findings of typhoid fever. The verification test, like other laboratory methods of an immunologic nature, does not lend itself to mass testing. Even with serodiagnostic tests having a record of use for over a generation, mass testing is acceptedly wrought with difficulties. Briefly, in the application of the verification test to the detection of a false positive reaction in a given case, it is assumed that thorough search for clinical evidence of syphilis had led to negative findings and that the examining physician had concluded that the patient was most likely free from syphilis. A verification report of a false positive reaction in a case such as this may thus be said to be corroborated by clinical findings.

THE "TYPING" OF SERODIAGNOSTIC REACTIONS

8. *Reporting Verification Results to Physicians.*—The function of the verification test is to type positive serodiagnostic reactions on the basis of their specificity. Hence, the results of the verification test are reported not as positive or negative, but only in respect to the specificity of the serodiagnostic reactions. To illustrate: (1) A physician receives a report of a positive serodiagnostic reaction in a given case. (2) He believes this reaction to be a false positive and requests a verification test. (3) The verification test determines whether the serodiagnostic reaction shows characteristics of a luetic type or of a nonluetic type of reaction or perhaps of both types.

9. *Types of Serodiagnostic Reactions Encountered With Verification Test.*—(1) "General biologic (nonluetic) type"* is the designation employed when, according to the verification test, a serodiagnostic reaction shows characteristics of a false positive. (2) "Type of reaction similar to that obtained in lues" is the designation employed when, according to the verification test, a serodiagnostic

*This designation, it is believed, is more desirable than the purely negative designation of "false positive," since in addition to indicating absence of syphilis it indicates the presence of a reaction which in many instances accompanies a pathologic disturbance or biologic imbalance. The term "general biologic" is being employed because of the biologically widespread nature of this reaction, especially among lower animals. The term we believe is also preferable to "biologic" in designating false positives, since syphilitic reactions or "true positives" are also biologic reactions.

The terms "lues" and "luetic" are employed instead of syphilis and syphilitic because the latter terms are too obvious to the laity.

reaction shows characteristics of a syphilitic reaction. (3) "Negative type" is the designation employed when the results of the verification test are negative. (4) "Inconclusive" is the term used when the verification results are atypical. (5) Occasionally, a positive serodiagnostic reaction embodies, according to the verification test, both the syphilitic and nonsyphilitic types of reactions.

10. *Procedures of the Verification Test.*—(1) The triple quantitative technique, described later, is employed in typing strongly positive (++++) serodiagnostic reactions on the basis of their specificity. Three types of reactions are generally encountered: the luetic type, the general biologic type, or a combination of both types. (2) The differential temperature technique, also described later, is employed in the typing of moderately and weakly positive (+++, ++, + and ±) serodiagnostic reactions. Here also three types of reactions are generally encountered: the luetic type, the general biologic type, and a combination of both types. (3) The salt dispersibility technique, due to its simplicity, is employed with all sera. (4) The standard Kahn test and a modified Kahn procedure (Method B) are also employed in performing verification tests.

THE VERIFICATION TEST IN KNOWN CASES OF SYPHILIS

11. *The Verification Test in the Various Stages of Syphilis.*—The vast majority of syphilitic cases give the luetic type of reaction with the verification test; a relatively small number give both the luetic type and the general biologic type. In a small percentage of cases of early syphilis, only the general biologic type of reaction may be encountered. In repeated examinations, as the serologic reaction becomes stronger in this stage of syphilis, the luetic type of reaction is obtained. This temporary occurrence of the general biologic type of reaction in early syphilis must be kept in mind when this type of reaction is reported in a case in which there is the slightest possibility of the presence of early syphilis. Repeated verification tests at intervals of a week or more are necessary. These tests will indicate the trend of the serodiagnostic results and will determine whether the general biologic type of reaction persists. In the various stages of syphilis other than early syphilis the luetic type of reaction is generally obtained.

12. *The Verification Test in Treated Cases of Syphilis.*—The common reaction obtained is of the luetic type. Occasionally, the general biologic type is obtained. Preliminary studies of verification reactions in treated cases have led to several observations which promise to prove of clinical value. Thus: (1) The general biologic type of reaction is occasionally encountered following treatment, in addition to the luetic type, in persons who presumably show a tendency to give the former type of reaction under conditions of pathologic disturbance. It is believed that the toxic effect of the antisyphilitic treatment may result in sufficient pathologic disturbance as to be the immediate cause of the general biologic type of reaction. If so, it is reasonable to assume that a higher degree of toxicity will call forth this type of reaction to a greater extent than a lower degree of toxicity. The verification test may thus indicate variations in toxicity of different methods of therapy. (2) The verification test in certain seropositive treated cases is capable of indicating in advance that they will in due time become seronegative. This finding is based on the

observation that the formed precipitates in verification reactions show a change in stability as these cases begin to approach seronegativity. The precipitates, at first stable and indispersible, become unstable and dispersible. The tendency toward dispersibility may become manifested weeks or months before seronegativity is reached. This capability of the verification test to indicate seronegativity in advance may be of value in the study of the efficacy of various methods of therapy. (3) The verification test in certain serologically fast cases following adequate therapy may establish that the reactions are not of the luetic type but of the general biologic type.

THE VERIFICATION TEST IN SERONEGATIVE CASES

13. *The General Biologic Type of Reaction in Seronegative Cases.*—Some persons who are clinically free from syphilis and who give negative serodiagnostic reactions, nevertheless give the general biologic type of reaction with the verification test. Indications are that these persons are "carriers" of false positives. They may be seronegative with one test but seropositive with another. Or they may be seronegative today and seropositive a week later. Then again, some nonsyphilitic persons, negative with serodiagnostic tests, are positive with supersensitive tests. Verification studies of persons giving negative serodiagnostic reactions reveal three main groups: (1) those giving the negative type of reaction, (2) those giving the general biologic type, and (3) a very small group of treated cases of syphilis giving the luetic type of reaction.

THE VERIFICATION TEST IN CASES IN WHICH FALSE POSITIVES ARE SUSPECTED

14. *Routine Applicability of Verification Test to Cases in Which False Positives Are Suspected.*—Experience gained with the verification test during the past several years indicates that the greatest value of the test lies in the investigation of the specificity of positive serodiagnostic (precipitation or complement fixation) reactions in such cases in which all clinical indications point to the absence of syphilis. When the verification test is applied to such cases, the general biologic type of reaction may be said to corroborate clinical findings, while the luetic type of reaction may be said not to corroborate clinical findings. It is with this view in mind that the interpretation of these two types of reactions is considered.

15. *Interpreting the General Biologic Type of Reaction.*—A single report of a general biologic (nonluetic) type of reaction can be interpreted as a false positive with reasonable safety, provided the donor gives a history of fluctuating or weak serodiagnostic reactions extending for some weeks or months; in the face of strongly positive serodiagnostic reactions, repeated verification studies are indicated to make sure that the results are persistently of the general biologic type. A general biologic type of reaction in the absence of a serologic history (i.e. when a person gives a positive serodiagnostic reaction presumably for the first time) should be checked preferably in a week or two before establishing a diagnosis of a false positive.

16. *Interpreting the Luetic Type of Reaction.*—A luetic type of reaction obtained in a case in which clinical indications point to a false positive requires additional clinical studies and repeated verification tests. In our experience, these

clinical and verification studies have led to the establishment of syphilis in most instances. In a few instances clinicians have expressed the belief that they were dealing with false positives in spite of persistent verification reports of the luetic type of reaction. A transient luetic type of reaction has been observed in an isolated nonsyphilitic case of postinfection or postvaccination, the reaction becoming of the general biologic type in the course of some weeks. Weakly positive serodiagnostic reactions, which according to the verification test are of the luetic type, are especially difficult to interpret. Repeated examinations at prolonged intervals are often necessary before a final diagnosis can be established.

PROCEDURES

1. Triple Quantitative Technique

Applicability.—This technique is applicable to sera which give strongly positive (++++) serodiagnostic (complement fixation or precipitation) reactions suspected of being false positives.

Technique.—The technique involves the use of three quantitative Kahn procedures: in the first, the serial dilutions of serum are made with distilled water; in the second, the serial dilutions are made with 0.9 per cent sodium chloride solution, and in the third, the serial dilutions are made with 2.5 per cent sodium chloride solution. Dilution ratios of 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50 are generally ample. Standard Kahn antigen suspension (prepared with 0.9 per cent salt solution) is used in 0.025 c.c. amounts, and each of the serial dilutions of serum in 0.15 c.c. amounts. After permitting the serum-antigen mixtures to stand for three to five minutes, the tubes are shaken for three minutes in the usual manner. Distilled water or salt solution of the concentration corresponding to that used in the serial dilutions of serum is then added to the tubes in 0.5 c.c. amounts; after agitating the tubes for about ten seconds to mix the ingredients, the precipitation results are read.

Results.—Known syphilitic sera generally give a higher quantitative titer with the serial dilutions of serum with 2.5 per cent salt solution than with 0.9 per cent salt solution (luetic type of reaction). Known nonsyphilitic sera generally give a higher quantitative titer with the serial dilutions of serum with 0.9 per cent salt solution than with 2.5 per cent salt solution (general biologic type of reaction).

The quantitative titer with the serial dilutions of serum with water throws additional light on the type of the reaction in question. Thus, this titer in a luetic type of reaction is lower, and in a general biologic type of reaction is higher, than the titer with 0.9 per cent salt solution, unless both the luetic and general biologic types of reactions are present. Then, the titer with the serial dilutions of serum with 0.9 per cent salt solution is lower than either with water and with 2.5 per cent salt solution. Table I presents typical reactions given by the triple quantitative technique.

Note.—Sera of low quantitative titer are also examined in this laboratory with the differential temperature technique. The sera are first diluted with an equal amount of 0.9 per cent salt solution or with half the amount (depending on the quantitative titer given by the serum) before employing the differential temperature technique.

2. Differential Temperature Technique

Applicability.—This technique is applicable to sera giving moderately and weakly positive (+++, ++, + and ±) serodiagnostic (complement fixation or precipitation) reactions, suspected of being false positives.

Technique.—(1) Kahn racks with test tubes are placed in a 37° C. water bath for fifteen minutes before performing the tests. The pipettes necessary in performing the test are kept at 37° C.* (2) The serum is heated for thirty minutes at 56° C. and is then placed in the 37° C. water bath for fifteen minutes before using. (3) Standard Kahn antigen is mixed with salt solution at room temperature in the usual manner according to the titer, and the antigen suspension is immediately transferred to a Kahn tube and placed in the water bath for

TABLE I

ILLUSTRATIVE RESULTS OF TRIPLE QUANTITATIVE TECHNIQUE

SERIAL DILUTIONS OF SERUM	PERCENTAGE OF SODIUM CHLORIDE SOLUTION EMPOLYED IN PREPARING THE SERIAL DILUTIONS OF SERUM		
	0*	0.9	2.5
	Luetic type of reaction		
1:5	+	++++	++++
1:10	-	++++	++++
1:20	-	++	++++
1:30	-	-	++++
1:40	-	-	++++
1:50	-	-	-
1:60	-	-	-
	General biologic (nonluetic) type of reaction		
1:5	++++	++++	±
1:10	++++	++++	-
1:20	++++	±	-
1:30	++++	-	-
1:40	++++	-	-
1:50	++	-	-
1:60	-	-	-
	Luetic and general biologic (nonluetic) types of reactions		
1:5	++++	++++	++++
1:10	++++	+	++++
1:20	++++	-	++++
1:30	++++	-	++++
1:40	++++	-	++++
1:50	±	-	++
1:60	-	-	-

*Distilled water. The same antigen suspension (prepared according to titer with 0.9 per cent salt solution) is employed with each of the serum dilutions.

fifteen minutes. (4) The amounts of antigen suspension (0.05, 0.025, and 0.0125 c.c.) are then deposited at the bottom of the Kahn tubes, followed by the serum (in 0.15 c.c. quantities), and the mixtures shaken for ten seconds without removing the rack from the water bath. The rack remains in the bath for ten minutes and is then shaken for three minutes at room temperature. (5) Physiologic salt solution kept in a 37° C. water bath is then added to each tube in the usual amount (1, 0.5, and 0.5 c.c.); the tubes are agitated sufficiently to assure proper mixing and the results are read immediately. (6) A microscope mirror or a slit lamp is used in the reading of results.

*Specially constructed water baths for this technique are supplied by A. S. Aloe Co., St. Louis.

The technique at 1° C. requires a refrigerated bath at 1° C., and if not available, an ice water bath with chopped ice. The steps in the technique as carried out in the 37° C. water bath are applied to the technique in the 1° C. water bath. The temperature of the serum and antigen suspension is brought to 1° C., and the serum is added to the antigen suspension and mixed at this temperature. Tests performed at 1° C. require special care in reading the results due to the film of moisture condensed on the outside of the tubes. This film disappears by dipping the tubes in a solution of cold alcohol. Not more than ten tests should be carried out at any one time, either at warm or cold temperatures.

Results.—Syphilitic sera generally give more marked precipitation at 37° C. than at 1° C. (luetic type of reaction), while nonsyphilitic sera generally give more marked precipitation at 1° C. than at 37° C. (general biologic type of reaction). Typical illustrations of these types of reactions are presented in Table II. It is noted that similar precipitation results at all temperatures are considered as inconclusive.

TABLE II

ILLUSTRATIVE RESULTS OF DIFFERENTIAL TEMPERATURE TECHNIQUE

REACTING TEMPERATURE								
37° C.			21° C.			1° C.		
TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3	TUBE 1	TUBE 2	TUBE 3
			Luetic type of reaction					
++++	++++	++++	-	++++	++++	-	±	++
			General biologic (nonluetic) type of reaction					
-	+	++	-	++++	++++	++++	++++	++++
			Inconclusive reaction					
-	++++	++++	-	++++	++++	-	++++	++++

TABLE III

ILLUSTRATIVE RESULTS OF SALT DISPERSIBILITY TECHNIQUE

FIRST READING OF RESULTS OF STANDARD KAHN TEST IN WHICH REACTION IS MODERATELY POSITIVE

Tube 1	Tube 2	Tube 3
±	++	++++

Addition of 30% NaCl solution c.c.

0.15	0.1	0.1

Shake rack 15 seconds and place for 5 minutes in water bath at 37° C

Luetic type of reaction—precipitates are not dispersible.

Tube 1	Tube 2	Tube 3
+	++	++++

General biologic (nonluetic) type of reaction—precipitates are dispersible.

Tube 1	Tube 2	Tube 3
-	-	-

3. Salt Dispersibility Technique

Technique 1.—When applied to weakly positive sera suspected of giving false positive (precipitation or complement fixation) reactions.

Procedure: A 30 per cent solution of sodium chloride of "reagent" quality is employed in this technique. The standard Kahn test is performed and the

results read in the usual way after the addition of 0.9 per cent salt solution to each tube. Immediately, 0.15 c.c., 0.1 c.c., and 0.1 c.c. of the 30 per cent salt solution are added, respectively, to the three tubes; the rack is then shaken by hand for fifteen seconds, placed in the 37° C. water bath for five minutes, again shaken for fifteen seconds, and the results read.

Results.—In the case of syphilitic sera, the precipitates are generally either the same as before the addition of the concentrated salt solution or they are more pronounced. In the case of nonsyphilitic sera, the precipitates become dispersed and the results appear negative. Table III presents typical reactions given by the salt dispersibility technique.

Technique 2.—When applied to all sera suspected of giving false positives and to the several verification techniques.

Procedure: Due to the simplicity of the dispersibility technique, it is being applied in this laboratory to the triple quantitative and differential temperature techniques in addition to its application to the standard Kahn technique. Thus, after reading the results of the triple quantitative technique, 0.1 c.c. of 30 per cent salt solution is added to each tube showing a precipitate, shaken fifteen seconds, incubated at 37° C. for five minutes, again shaken for fifteen seconds, and the final results read. Similarly, after reading the results of the differential temperature technique, 0.15 c.c., 0.1 c.c., and 0.1 c.c. of the 30 per cent salt solution are added, respectively, to each set of the three tubes showing precipitates and the test is completed as in Table III.

Results.—As a general rule, precipitates in nonsyphilitic reactions become dispersed on the addition of the 30 per cent salt solution, while precipitates in syphilitic reactions do not become dispersed. In early syphilis and in highly treated cases of this disease, dispersibility of the precipitates may be noted. But in the absence of clinical indications of syphilis, the dispersibility of precipitates indicates false positive reactions.

Discussion of results.—(1) *Dispersibility technique applied to four plus Kahn reactions.* A definite relationship exists between the strength of nonspecific serologic reactions and the concentration of salt solution required for the dispersion of precipitates. Since different four plus reactions may be of different quantitative titers, the precipitates in such reactions may require different salt concentrations for their dispersion. In the case of nonsyphilitic sera of high titer, precipitates which are not dispersed in the standard Kahn test by the usual dispersibility technique will often disperse by a second addition of 0.15 c.c., 0.1 c.c., and 0.1 c.c. of 30 per cent salt solution to the tubes. In some instances even this second amount of concentrated salt solution will not disperse the precipitates of the strongly positive reactions. Hence, nondispersibility of the precipitate in a strongly positive reaction cannot by itself be interpreted as a syphilitic type of reaction. The dispersibility of the precipitate is a common property of weak nonsyphilitic reactions and is occasionally noted also in nonsyphilitic reactions of high titer.

(2) *Dispersibility technique applied to the triple quantitative procedure.* Generally precipitates in syphilitic sera do not disperse on the addition of 30 per cent salt solution. Now and then a serum is encountered which gives a higher quantitative titer in the serum dilutions with 2.5 per cent salt solution than in the serum dilutions with 0.9 per cent solution (suggesting a luetic type of reac-

tion), but which shows dispersible precipitates in all tubes on the addition of the 30 per cent salt solution. It is believed that such dispersibility is strongly suggestive of a false positive reaction. In instances in which only the precipitates in the highest serum dilutions show dispersibility while the precipitates in the low serum dilutions do not show dispersibility, the reactions are generally of the syphilitic type, noted particularly in treated cases.

(3) *Dispersibility technique applied to the differential temperature procedure.* Dispersibility of precipitates in reactions obtained at 37° and 1° C. is the common picture presented by false positives. In syphilitic cases, the precipitates are generally not dispersed in reactions obtained at 37° C. but are dispersed in reactions obtained at 1° C., although they may not be dispersed also in the latter reactions.

4. Method B

Method B consists of performing the standard Kahn test as usual with the exception that, after the three-minute shaking period, sodium chloride solution of 2.5 per cent concentration instead of 0.9 per cent is added to the tubes in the regular amounts of 1.0, 0.5, and 0.5 c.c., respectively. When this method is applied to doubtful serodiagnostic reactions, it is frequently observed that in nonluetic cases the results are weaker than those given by the standard Kahn test, while in luetic cases the results are the same or stronger.

5. Combined Use of the Several Verification Techniques in Practice

When a blood specimen reaches this laboratory for a verification test, the serum is separated from the clot in the usual way, checked for clarity, heated for thirty minutes at 56° C., rechecked for clarity, and within about ten minutes after the heating period, the following preliminary tests are performed:

1. *The standard Kahn test.*

2. *Method B,* which consists of the standard Kahn test, in which the diluent added to the tubes after the three-minute shaking period (1.0, 0.5 and 0.5 c.c.) consists of 2.5 per cent salt solution instead of 0.9 per cent.

3. *Salt dispersibility technique.* The results of the above two tests are read and recorded, and without waiting fifteen minutes for rereading, 0.15 c.c., 0.1 c.c., and 0.1 c.c. of 30 per cent sodium chloride solution are added respectively to the three tubes of each test, shaken fifteen seconds, placed for five minutes in a 37° C. water bath and for two to three minutes at room temperature, shaken again for about fifteen seconds and the results read and recorded.

The results of the above three tests are taken into consideration before proceeding with the additional techniques.

In the case of weakly positive reactions: If Method B gives weaker results than the standard Kahn test, it is indicative of a false positive reaction; and in such a case, the salt dispersibility technique will lead to dispersion of the precipitates and to negative results. The differential temperature technique is then performed for confirmation and is likely to give a nonluetic type of reaction. If Method B gives results which are the same or stronger than those of the standard test, the precipitates are generally not dispersible and the differential temperature technique is likely to give a luetic type of reaction. The application of the dispersibility technique to completed differential temperature reactions commonly gives additional confirmation of either the luetic or nonluetic types, as the case may be.

In the case of strongly positive reactions: The results of the standard Kahn test and of Method B are equally four plus and the precipitates may or may not be dispersed by the concentrated salt. The triple quantitative technique is then performed, the results read, and the salt dispersibility technique applied to the completed reactions. Typically nonsyphilitic patterns with the triple quantitative technique are generally confirmed by complete dispersion of the precipitates. Inconclusive patterns with the triple quantitative technique which show dispersibility of the precipitates indicate nonsyphilitic type of reactions. Rarely one encounters a syphilitic pattern in which the precipitates become dispersed. It is believed that in such instances (in the absence of clinical evidence of syphilis) the reaction is in all probability of the nonluetic type.

In the case of negative reactions: The differential temperature technique is employed to determine if a reaction is obtained at 1° C., generally given by "carriers" of false positives, or at 37° C., occasionally obtained in highly treated cases of syphilis.*

AN EXPLANATORY STATEMENT TO PHYSICIANS ON VERIFICATION REPORTS

In this laboratory, the following statement on interpretation is submitted to physicians with each verification report:

The verification test is a supplementary procedure to serodiagnostic tests; its function is to "type" positive serologic (precipitation or complement fixation) reactions on the basis of their specificity; in practice, the test is applicable to cases in which physicians suspect false positive reactions with complete lack of clinical indications of syphilis.

1. "*Type of reaction similar to that obtained in lues*" is reported when, according to the verification test, the positive serodiagnostic reaction shows characteristics of a specific reaction. This type of reaction is commonly noted in known cases of syphilis.

2. "*General biologic (nonluetic) type of reaction*" is reported when, according to the verification test, the positive serodiagnostic reaction shows characteristics of a false positive. This type of reaction is commonly noted in nonsyphilitic cases.

(1) *General biologic (nonluetic) type of reaction in seronegative cases.* Certain nonluetic persons, although giving negative reactions with serodiagnostic tests, may give the general biologic type of reaction with the verification test. These persons are looked upon as potential "carriers" of false positive reactions, giving such reactions off and on with serodiagnostic tests and more frequently with supersensitive tests.

(2) *General biologic (nonluetic) type of reaction in treated cases of syphilis.* Persons who have a tendency to give false positive reactions may exhibit the same tendency after they contract syphilis, and the verification test will then demonstrate the presence of both the luetic and general biologic types of reactions. Following therapy, as these persons are approaching seronegativity, they may give the general biologic type only.

3. "*Negative type*" is reported when the results of the verification test are negative. This type of reaction usually accompanies negative serodiagnostic reactions.

4. "*Inconclusive*" is reported when the verification results are atypical.

5. *Exceptions:*

(1) In isolated instances, physicians have claimed nonspecificity for a given positive serodiagnostic reaction in spite of persistent verification findings of the luetic type.

(2) Transient reactions of the luetic type have been observed in a small percentage of seropositive (nonluetic) cases following infection or immunization.

(3) Transient reactions of the general biologic type have been observed in a small percentage of known cases of early syphilis.

---

*Studies of the verification test with supersensitive antigens will be reported at a later date.

Interval retesting. Whenever verification results do not conform to clinical opinion or whenever there is a question of the diagnosis of early syphilis, it is of importance to repeat the verification test at intervals of weeks instead of days. Interval retesting will establish the serologic trend in questionable cases with far greater dependability than immediate retesting.

SUMMARY

Nonspecific and specific serologic reactions show similar characteristics with modern serodiagnostic (precipitation and complement fixation) tests for syphilis; hence, these tests do not offer a practical means for the detection of false positives in routine serologic practice. Studies carried out in this laboratory on differential characteristics of nonspecific and specific serologic reactions led to the observation that these reactions have different temperature and electrolyte requirements for optimal reactivity. This observation, in turn, led to the development of several procedures capable of determining whether certain serodiagnostic reactions possess characteristics of specific or nonspecific reactions, or of both types of reactions. These procedures (termed verification test) "type" serodiagnostic reactions on the basis of their specificity and are of practical value, especially when applied to the typing of seropositive cases that are free from clinical indications of syphilis. When applying the verification test to such cases, nonluetic type of reactions may be looked upon as being corroborated by clinical opinion, and the diagnosis of "false positive" can then be made with reasonable certainty.

REFERENCES

1. Kahn, R. L.: A Serologic Verification Test in the Diagnosis of Latent Syphilis, Arch. Dermat. & Syph. 41: 817, 1940.
2. Escobar, J. J.: La Reaccion de Verificaccion de Kahn en el Carate, Boletin Clinico 6: 543, 1940.
3. Kahn, R. L.: False Positive Reactions in Serology of Syphilis, Univ. Hosp. Bull., Ann Arbor 6: 26, 1940.
4. Moore, J. E., Eagle, H., and Mohr, C. F.: Biologic False Positive Tests for Syphilis III. A Suggested Method of Approach to Their Clinical Study, J. A. M. A. 115: 1602, 1940.
5. Chargin, L., and Rein, C. R.: The Kahn Verification Test, An Appraisal of the Test Based on Clinical and Serologic Evidence, Arch. Dermat. & Syph. 44: 1031, 1941.
6. Kahn, R. L., McDermott, E. B., and Marcus, S.: Effect of Temperature on Kahn Reaction: I. With Serologically Positive Sera of Lower Animals, II. With Serologically Positive Sera of Human Syphilis, III. With Serologically Positive Sera in the Absence of Syphilis, IV. With Serologically Negative Sera in the Absence of Syphilis, Am. J. Syph., Gonor. & Ven. Dis. 25: 151, 157, 162, 173, 1941.
7. Jacobsthal, E.: Sobre el principio del "verification-test" de Kahn en el paludismo y su simplificación en las reacciones de Kahn y Collier Jacobsthal-Padilla, Bol. san. Guatemala 12: 190, 1941.
8. Thomas, G. E., and Garrity, R. W.: Routine Kahn Blood Reactions, Report of 10,000 Tests Made on Naval Recruits, U. S. Nav. M. Bull. 39: 72, 1941.
9. Lynch, F. W., Boynton, R. E., and Kimball, A. C.: False Positive Serologic Reactions for Syphilis, Due to Smallpox Vaccinations (Vaccinia), J. A. M. A. 117: 591, 1941.
10. Kahn, R. L.: A New Verification Method in Serology of Syphilis, Univ. Hosp. Bull., Ann Arbor 8: 45, 1942.
11. Carter, B. B.: The Use of a Quantitative Test in Verification Procedures, Am. J. Syph., Gonor. & Ven. Dis. 26: 629, 1942.
12. Green, M. N., and Forster, G. F.: The Sensitivity and Specificity of the Kahn Reaction in the Temperature Range From 37° C. Through 56° C. With Syphilitic Sera. With a Consideration of the Value of the Kahn Verification Test in Syphilis, Am. J. Syph., Gonor. & Ven. Dis. 26: 632, 1942.
13. Kahn, R. L.: Serology in Syphilis Control, Baltimore, 1942, Williams and Wilkins Company, p. 166.
14. Green, M. N., and Shaughnessy, H. J.: Effect of Electrolytes Upon Kahn Precipitates From Human and Animal Sera, Proc. Soc. Exper. Biol. & Med. 51: 287, 1942.
15. Kahn, R. L., Marcus, S., McDermott, E. B., and Adler, J.: A Serologic (Nonsyphilitic) Reaction Approaching Universal Sensitivity, J. Investig. Derm. In press.

CLINICAL AND EXPERIMENTAL

THE PRODUCTION OF CHRONIC HYPERTENSION IN DOGS BY PROGRESSIVE LIGATION OF ARTERIES SUPPLYING THE HEAD*

H. R. Fishback, Sc.D., M.D., Frank E. Dutra, M.S., M.D., and Edwin T. MacCamy, M.S., M.D., Chicago, Ill.

THE puzzle of the etiologic relationships of high blood pressure has provided the impetus for quite varied and extensive research. The trend of this work has changed through the years, but there have been two conditions under investigation quite constantly; namely, arteriosclerosis and kidney disease.

While arteriosclerosis is commonly found with chronic hypertension, it is not invariably demonstrable. Furthermore, there may be quite extensive sclerosis, especially of the large vessels, without elevation of the blood pressure. Sclerosis of arterioles has been stated to be quite generally present in chronic hypertension. Gull and Sutton[1] believed this to be the essential pathologic change, alterations of kidneys or other structures being considered as secondary to it. Several studies on the distribution of arteriosclerosis have been made on autopsy material with varied interpretations of the significance of the findings.[2, 3, 4]

Clinical investigation has been carried out by sections of specimens from the skeletal muscles,[5] from the standpoint of correlating sclerosis of arterioles with hypertension. Study of the retinal vessels is easily carried out, and often the changes found are not only diagnostic of hypertension but may indicate definitely the type of hypertension.[6] Such changes may be found to indicate a former hypertension even though the pressure at the time of examination is found to be lowered.[7]

Pathologists have long noted, at autopsies of individuals with hypertension that gross evidence of arteriosclerosis often is marked in one organ and absent or only slight in other organs. Sclerosis of the aorta or its large branches is not necessarily associated with organ changes. The three important structures involved frequently are the kidneys, brain, and heart. In explanation of this association it was usually said that these were "vital" organs and that arteriosclerosis in any such organ was accompanied by increased blood pressure, in order to drive sufficient blood through it to maintain life.

This simple mechanistic explanation is disputed by the work of Page[8] and his co-workers, who interpret their results as indicating the formation of the specific substance "renin" by a damaged kidney, the renin then being changed by a blood activator into the effective antiotonin, which increases blood pres-

*From the Department of Pathology, Northwestern University Medical School, Chicago, Illinois.

Received for publication, October 31, 1942.

sure. As a result of his extensive and important work on hypertension, Goldblatt,[9] also, believes that the initiating principle derives from the kidneys exclusively.

Only one short note[10] has been found concerning the production of chronic hypertension by operative reduction of blood flow to the brain. Observations of the acute rise of blood pressure with sudden decrease of the cerebral blood supply[11] have long been on record. The injection of kaolin into the cisterna magna has been found to result in hypertension.[12] This is believed to be associated with a communicating type of hydrocephalus, with impaired drainage of the cerebrospinal fluid through the perineural and perivascular lymphatics, although in rats the fluid content of the brain tissue was not increased.[13]

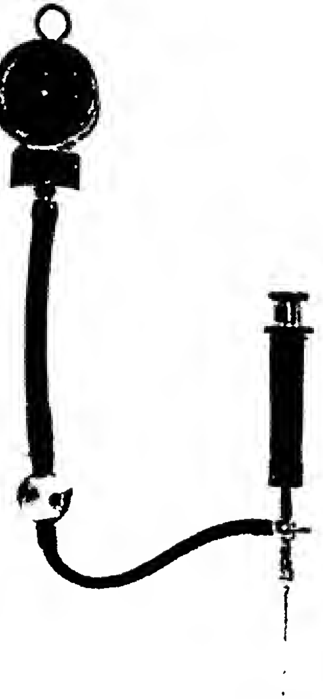


Fig. 1.

We have been interested, over a period of four years, in the effect on the blood pressure of a series of arterial ligations in dogs planned to lessen the blood supply of the brain. The ligations were done in stages, the sequence of operations being changed from time to time. The total series included the ligation of both common carotids in the region of the sixth cervical vertebra, and their dissection upward including the dissection, ligation, and excision of all branches as far out as possible from the bifurcation. Both carotid sinuses were completely removed. Also, the vertebral arteries were ligated just distal to their points of origin, and in the suboccipital region just before they enter the vertebral canal. Afterwards, various exploratory operations were performed for the ligation of any anomalous or collateral arteries which could be found.

As might be expected, the mortality in such a long series of operations was quite high, but decreased with knowledge of when and how to do the ligations. A total of 47 dogs has been used, with 10 of them successfully carried through the series.

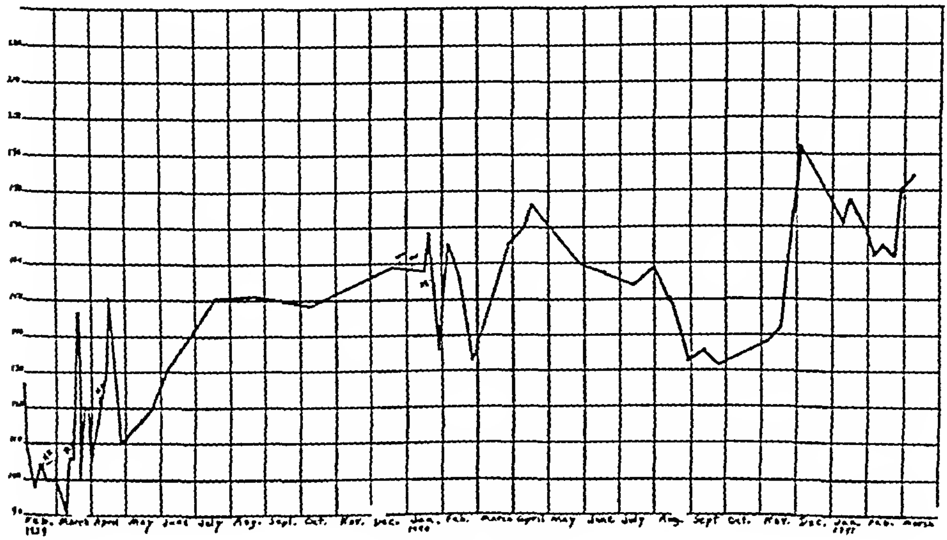


Fig. 2.—Dog 1.

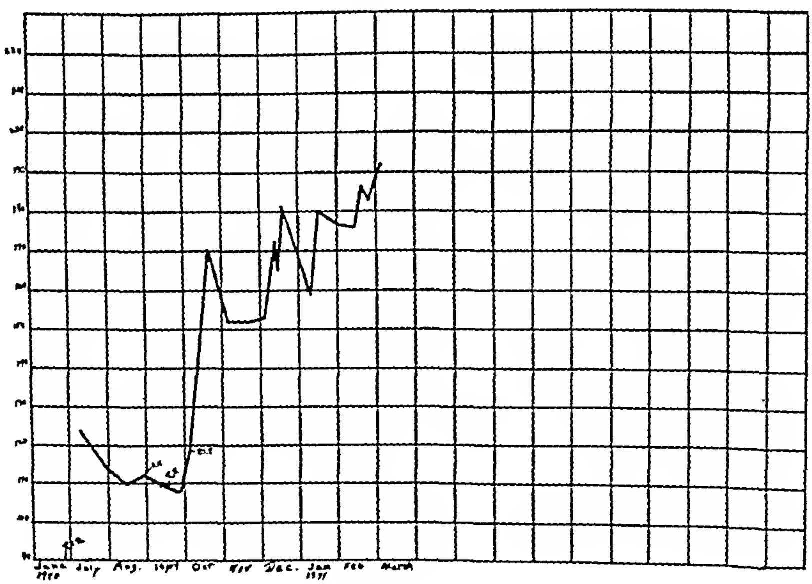


Fig. 3.—Dog 2.

Blood pressures were taken by direct puncture of the femoral artery with a twenty-gauge needle connected through a three-way valve with a glass syringe and an aneroid manometer (Fig. 1). Five per cent sodium citrate solution was drawn up into the syringe and forced out into the rubber tube, so as to fill the glass bulb about one-third full and raise the manometer pressure to about the expected blood pressure of the animal. With the stopcock

turned so as to allow blood to show in the citrate of the syringe, the needle was inserted into the femoral artery and the stopcock then turned to connect the needle with the pressure system. The manometer pointer oscillated from four to six points on the average, and the midpoint of the oscillations was taken as

Fig. 4.—Dog 4.

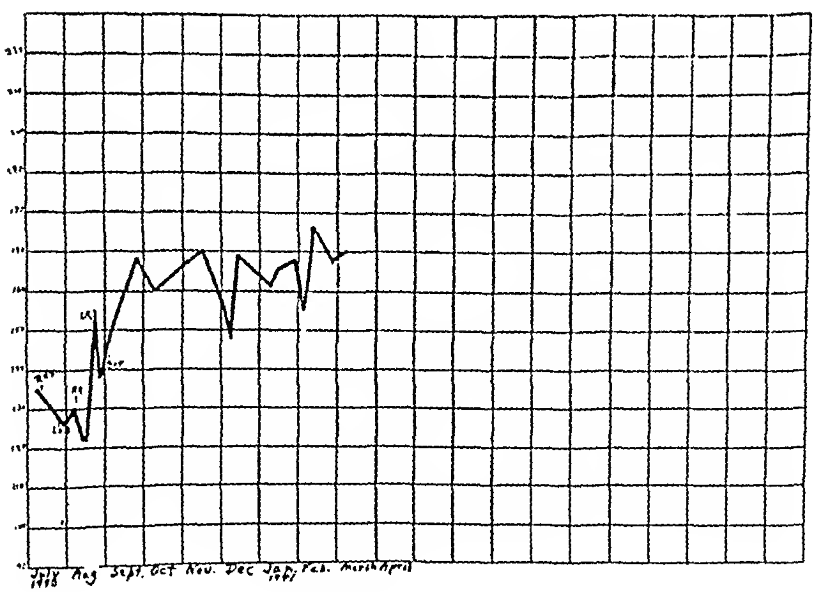


Fig. 5.—Dog 5.

the mean blood pressure. With a period of training the dogs became accustomed to the puncture and would lie quietly while it was carried out. Large dogs, usually of the police dog breed, were used.

The type of blood pressure response to the ligations, together with the time relationships, are shown in the accompanying graphs. Eight of the ten animals carried through the operative series showed a definite, and some of these a marked, rise of blood pressure. This ranged from controls of 100 to

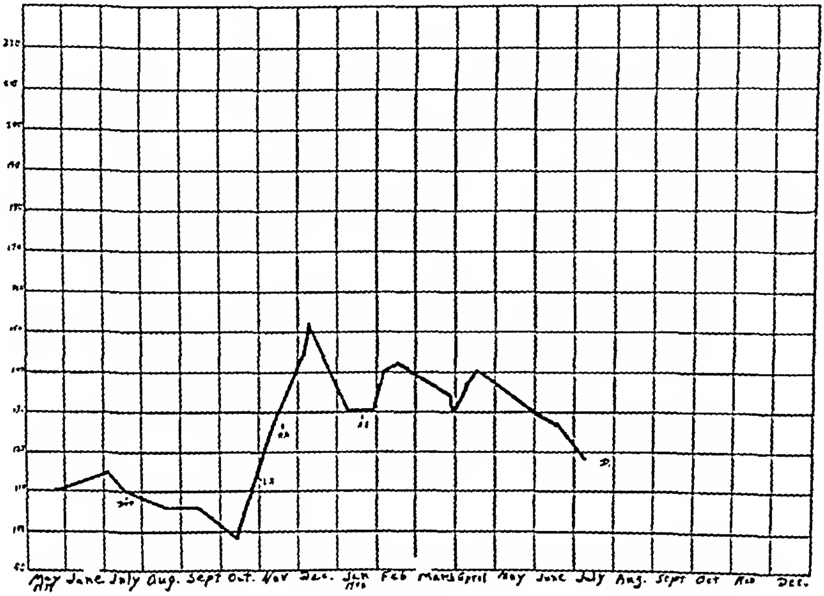


Fig. 6.—Dog 7.

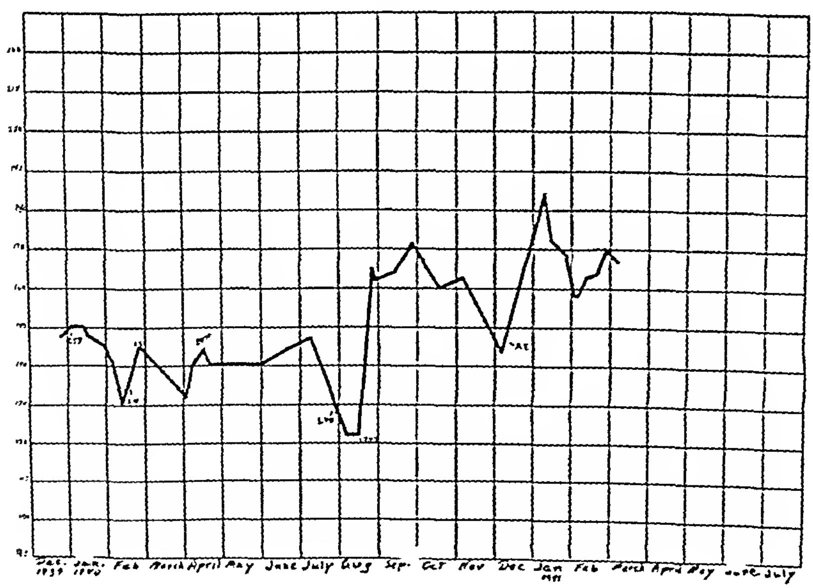


Fig. 7.—Dog 8.

120 mm. Hg usually, up to high points somewhat over 190 mm. Hg. The graphs illustrate the somewhat irregular curves, with variables up to 20 mm. Hg. Such variations occurred from day to day, often for no accountable reason. Two of the ten dogs showed no sustained rise of pressure.

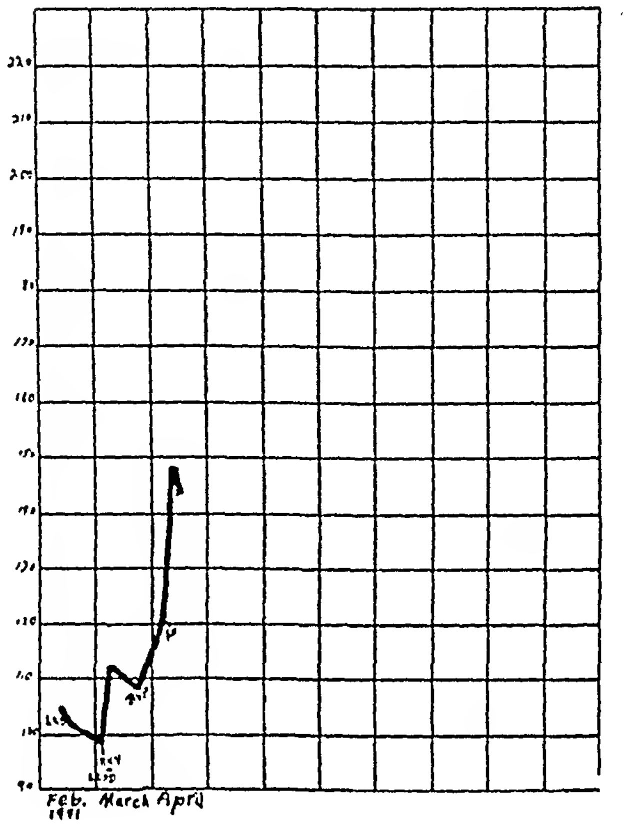


Fig. 8.—Dog 9.

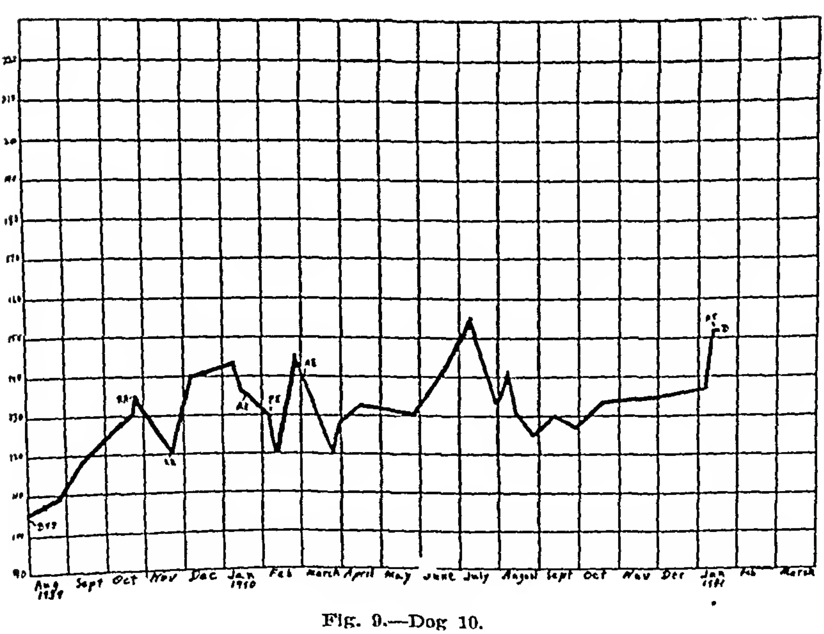


Fig. 9.—Dog 10.

Several workers have reported acute experiments with the production of hypertension by section of the aortic depressor nerves[14, 15, 16, 17] together with denervation of the carotid sinuses. Chronicity of this hypertension has been reported,[18, 19, 20] but others[21, 22] have failed to produce chronic hypertension by this means; this result is shown in our work also.

In four of our ten animals we completely removed the sinuses before the vertebral arteries were sectioned in the occipital region, so that the circulation to the brain was not diminished by their removal. In the graphs of Dogs 2 and 4 the blood pressure is shown to be uninfluenced by removal of the carotid sinuses, whereas hypertension was established later, after the vertebral arteries had been occluded in the occipital region. Experiment on Dog 8 was started by denervation of both carotid sinuses as a single operation. This was done on each side by stripping all nervous and fascial tissue from the common carotid and its branches for a distance of two or more cm. from the bifurcation in all directions. No rise of blood pressure occurred within six weeks. Over a period of five months the series of ligations was performed on this dog, which developed a heightened blood pressure a week after the last operation. Experiment on Dog 9 was begun with removal of the right carotid sinus, complete denervation of the left one, and ligation of the left cranial thyroid artery. No rise of blood pressure occurred within two weeks. Both vertebrals were then tied in the occipital region. After two more weeks both vertebrals and both common carotids were operated low in the neck, and a pronounced blood pressure increase developed.

We believe that denervation or removal of the carotid sinuses has no sustained effect on the blood pressure.[23]

The rise of blood pressure which occurs after multiple cerebral artery ligations appears to be directly associated with the interference with cerebral circulation.

In the dog, cerebral ischemia is difficult to achieve and still more difficult to maintain. Andreyev[24] has studied the development of cerebral collaterals formed after interruption of the major arterial supply to the head. By injection and x-ray he demonstrated quite strikingly the large number of collaterals promptly formed and the arteries involved in their formation. Multiple exploratory operations on our dogs revealed that newly formed arteries developed with a falling blood pressure curve. We are led to believe that once a high blood pressure has been developed by our method, a sustained drop of pressure in an otherwise healthy dog indicates the formation of collateral channels supplying an adequate amount of blood to the brain.

The mechanism by which hypertension is produced by arterial-ligation ischemia of the brain is not known. Either a nervous mechanism or a humoral one may be active. Anrep and Starling[25] demonstrated that a nervous mechanism can initiate hypertension in acute cerebral ischemia. This hypertension was prevented by section of the cord between the vasomotor centers and the sympathetic outflow. The humoral factor had been ruled out.

Page and his co-workers have brought forward evidence to indicate that constriction of the renal artery causes the kidney to produce a humoral mediator of hypertension.[6] Similarly, the hypertension produced by cerebral artery ligation may result from a hypertensive substance formed in the ischemic

brain. To date no work bearing on the presence or identity of such a substance has been performed.

SUMMARY

Chronic hypertension has been produced in dogs by ligation, in series, of the arteries supplying the head.

Removal or manipulation in situ of the carotid sinuses alone has no prolonged effect on the blood pressure of dogs.

REFERENCES

1. Gull, W. W., and Sutton, H. G.: On the Pathology of the Morbid State Commonly Called "Chronic Bright's Disease With Contracted Kidneys," M. Chir. Tr. 55: 273, 1872.
2. Moritz, A. R., and Oldt, M. R.: Arteriolar Sclerosis in Hypertensive and Nonhypertensive Individuals, Am. J. Path. 13: 679, 1937.
3. Bell, E. T., and Clawson, B. J.: Primary (Essential) Hypertension: A Study of Four Hundred and Twenty Cases, Arch. Path. 5: 939, 1928.
4. Fishberg, A. M.: Anatomic Findings in Essential Hypertension," Arch. Int. Med. 35: 650, 1925.
5. Kernohan, J. W., Anderson, E. W., and Keith, N. M.: The Arterioles in Cases of Hypertension, Arch. Int. Med. 44: 395, 1929.
6. Moore, F.: The Retinitis of Arteriosclerosis, and Its Relation to Renal Retinitis and to Cerebral Vascular Disease, Quart. J. Med. 10: 29, 1916.
7. O'Hare, J. P., and Walker, W. G.: Arteriosclerosis and Hypertension, Arch. Int. Med. 33: 343, 1924.
8. Helmer, O. M., and Page, I. H.: Purification and Some Properties of Renin, J. Biol. Chem. 127: 757, 1939.
9. Goldblatt, H.: Experimental Hypertension Induced by Renal Ischemia. The Harvey Lectures, May 19, 1938.
10. Nowak, S. J. G., and Walker, I.: Experimental Studies Concerning the Nature of Hypertension, New Eng. J. Med. 220: 269, 1939.
11. Hill, Leonard: The Physiology and Pathology of the Cerebral Circulation, London, 1896, Churchill.
12. Dixon, W. E., and Heller, H.: Experimentelle Hypertonie durch Ehröhung des intrakraniellen Druckes, Arch. f. exper. Path. u. Pharmakol. 166: 265, 1932.
13. Griffith, J. O., Jr., and Roberts, E.: Further Studies in the Mechanism of Vascular Hypertension Following the Intracisternal Injection of Kaolin in the Rat, Am. J. Physiol. 124: 86, 1938.
14. Hering, H. E.: Die Karotissinusreflexe auf Herz und Gefässe vom normal-physiologischen, pathologisch-physiologischen und klinischen Standpunkt, Dresden. Th. Steinkopf. 1927.
15. Koch, E., und Mies, H.: Chronischer arterieller Hochdruck durch experimentelle Dauerausschaltung der Blutdruckzügler, Krankheitsforschung. 7: 241, 1929.
16. Heymans, C., and Bouckaert, J. J.: Observations Chez le Chien en Hypertension Artérielle Chronique et Expérimentale, Compt. rend. Soc. de biol. 106: 471, 1931.
17. Dautrebande, L.: Réactions Vaso-Motrices à L'Oxygéne et a L'Acide Carbonique Chez le Chien en Hypertension Artérielle par Enervation des Zones Vasosensibles, Arch. Internat. Pharmacodyn. et de Thèrapie 40: 107, 1931.
18. Koch, E.: Ergebnisse der Kreislaufforschung. Bd. I. Die reflektorische Selbststeurung des Kreislaufes, Th. Steinkopf, Dresden, 1931.
19. Heymans, C., Bouckaert, J. J., and Regniors, P.: Les Sinus Carotidien et la Zone Homologue Cardioaortic, Doin, Paris, 1933.
20. Nowak, S. J. G.: Chronic Hypertension Produced by Carotid Sinus and Aortic-Depressor Nerve Section, Ann. Surg. 111: 102, 1940.
21. Green, M. F., DeGroat, A. F., and McDonald, C. H.: Observations on Denervation of the Carotid Sinuses and Section of the Depressor Nerves as a Method of Producing Arterial Hypertension, Am. J. Physiol. 110: 513, 1935.
22. Koch, E., and Mattonet, K.: Versuche zur Frage der Arteriellen Hypertonie nach Dauerausschaltung von pressoreceptorischen Kreislaufnerven, Ztschr. f. d. ges. exper. Med. 94: 105, 1934.
23. Goldblatt, H.: The Effect of Excision of the Carotid Sinuses on Experimental Hypertension Produced in Dogs, J. Exper. Med. 2: 175, 1940.
24. Andreyev, L. A.: Functional Changes in the Brain of the Dog After Reduction of Cerebral Blood Supply, Arch. Neurol. and Psychiat. 34: 481, 1935.
25. Anrep, G. V., and Starling, E. H.: Central and Reflex Regulation of the Circulation, Proc. Roy. Soc. 97B: 463, 1925.

THE HUMAN SKIN AS A CONDUCTOR OF 60-CYCLE ALTERNATING CURRENT OF HIGH INTENSITY, STUDIED ON "ELECTROSHOCK" PATIENTS*

HANS LÖWENBACH, M.D., AND JASPER E. MORGAN, PH.D., DURHAM, N. C.

THE human skin as a conductor of alternating currents has been the subject of many studies,[1] and it was soon shown that it does not act like any ordinary conductor, either metallic or electrolytic. These studies, however, were limited to currents of ten to fifteen milliamperes or less, partly because of the pain produced and partly from fear of causing lasting damage. While currents of higher intensity have been used on the skin of corpses, these experiments were unsatisfactory because of the probability that living and dead skins behave differently. Some information has come from industrial and domestic accidents, but it is far from precise and is frequently vitiated by erroneous computations.[2]

A method has been recently developed of treating mental patients with strong electric shocks applied directly to the head, so as to produce a minor or major convulsion.[3] This has provided an opportunity for extending these experiments and making more precise measurements.

METHODS

The apparatus used for treatment is very simple, consisting of a mechanical relay to make and break the circuit, the timing of which is controlled by a condenser.[4] Round copper plates, 5 cm., serve as electrodes and are attached to the head with even pressure, after a small amount of electrode jelly (sodium chloride, glycerine, tragacanth, and water) has been rubbed into the skin. In some experiments the jelly was omitted. In the first series of experiments the skin resistance of 31 patients was observed during 98 single treatments. Before the "shock" current was passed, a potential difference of about 20 microvolts was applied through the electrodes, and the resulting current was recorded with an ink-writing oscillograph. The impedance, that is, the potential difference divided by the current, was easily determined from a calibration chart.

The impedance-testing circuit was then disconnected, and a voltage varying from 50 to 120 volts was applied through the same electrodes for intervals of 1/10 to 4/10 of a second. The "shock" current was recorded by another ink-writing oscillograph during the time of current flow. The ink-writing oscillographs were adapted from the Grass electroencephalograph by using only the final power amplifier for each section.

After the relay had broken the current, twenty microvolts were again applied to the electrodes as quickly as a hand-operated switch would allow. The impedance was then recorded for various times, sometimes extending over 15 minutes. In a second series of experiments, the "shock" current was recorded by a Matthews oscillograph capable of following frequencies up to 15,000 cycles. This was done in order to see whether the form of the current at any time deviates from the sine form.

---

*From the Department of Neuropsychiatry and the Department of Physiology, Duke University School of Medicine, Durham, N. C.
Received for publication, October 24, 1942.

RESULTS

In agreement with previous workers who made observations with ballistic instruments,[5] we find that the current resulting from the application of voltages between 50 and 120 is much higher than expected from the impedance originally found. Almost immediately after the current is made, the impedance falls and remains at a low level during the entire time of current flow.

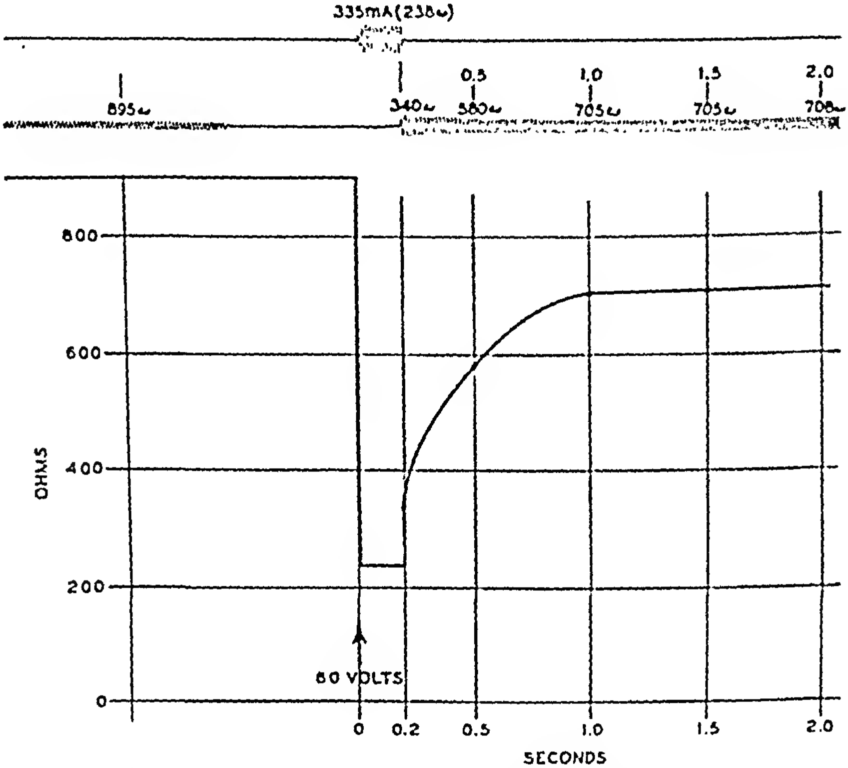


Fig. 1.—Skin resistance to 80 volts A.C. *Upper part:* Original tracings of skin resistance to 20 microvolts before and after "shock" and tracing of current during "shock." *Lower part:* resistance curve as derived from these tracings.

If the initial impedance is 2,000 ohms or less, it drops to a value between 300 and 120 ohms (Fig. 1). The exact value depends on how many volts are given, the lower values occurring after higher voltages. When the current is broken, the impedance rises again. Frequently there is an initial sudden rise of the impedance of 80 to 150 ohms, but in other cases the rise takes place gradually. It then increases slowly over a period of many seconds, and finally approaches the original value asymptotically. After lower tensions the pre-"shock" impedance returns within a shorter interval of time than after higher ones. A second shock, given before the impedance has reached the original value, produces the same current as the previous one; that is, no further drop of the impedance occurs. If all other components are the same, the drop occurs almost instantaneously, and during the next 2 to 3 cycles only a slight further decrease takes place (Fig. 2*b*). If, however, the resistance is higher than 2,000 ohms to begin with, as for example when no electrode jelly is being used, the

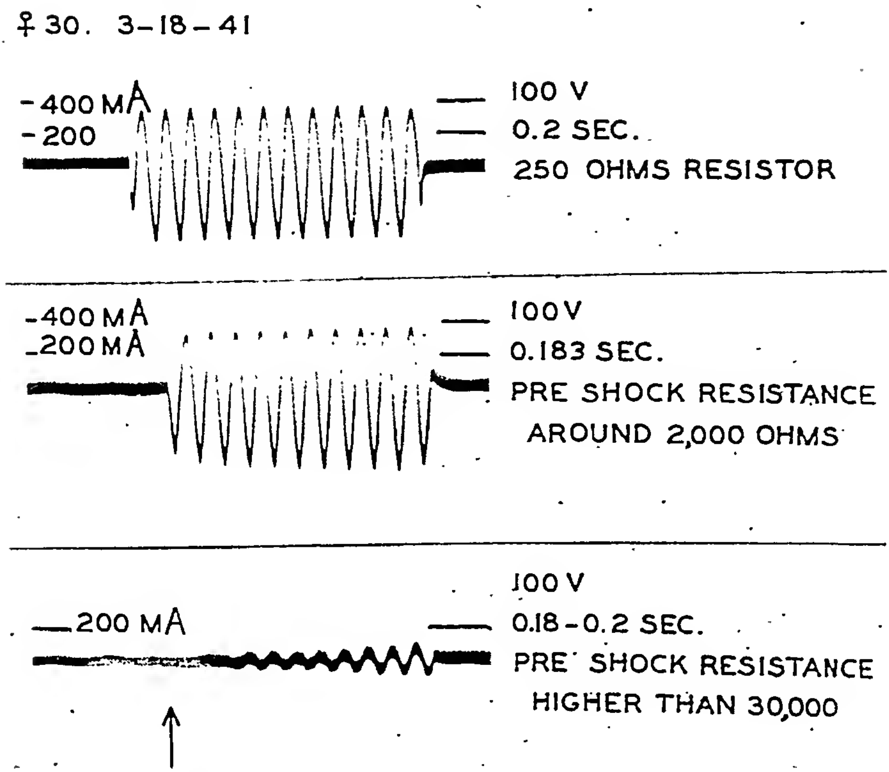


Fig. 2.—Oscillograms (Matthews' oscillograph) of 100 volts A.C. (*a*) passing through 250 ohm wire-wound resistance; (*b*, *c*) passing through human skin.

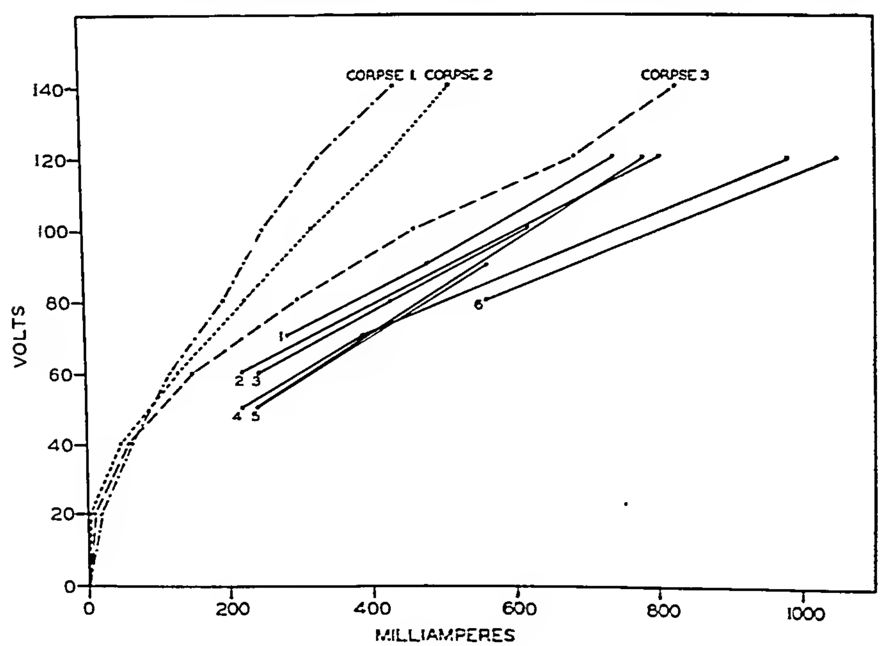


Fig. 3.—Voltage/current for 6 patients and for 3 corpses. Corpse 1 had been dead for 90 hours; corpse 2, for 24 hours; corpse 3, for 3 hours. The drop of the skin resistance when higher tensions are applied is larger in the living skin than in the dying skin. The voltage/current curve of the latter tends to approach a straight line.

current increases very gradually, and assuming the same time of current flow, it may never reach the same high level as otherwise (Fig. 2c).

No deviation from the sine form was observed in these experiments, and a few recordings with a cathode ray oscillograph point in the same direction.

The living skin then behaves like an electrolytic condenser which breaks down when tensions above 50 volts are applied. The impedance of the uninjured dying skin of the corpse is of the same order of magnitude as that of living skin for tensions up to 50 volts. If higher tensions are applied, the impedance of dying skin does not drop to such low values as are observed under living conditions. The longer the time after death, the less the drop, until finally the voltage/current curve approaches a straight line (Fig. 3).

Thus, it seems obvious that the sometimes elaborate attempts to measure the resistance of the skin before an electric shock treatment are not necessary. With electrode jelly and the electrodes in good contact, the resistance will usually be below 2,000 ohms and, during the time of current flow, will be between 150 and 250 ohms.

SUMMARY

The reactions of the living human skin as a conductor of 60-cycle alternating current up to 1,000 milliamperes was studied on 31 mentally ill individuals in 98 experiments. When 50-120 volts are given for 0.1-0.4 sec., the impedance drops instantaneously to a value between 270 and 120 ohms regardless of the original impedance, provided this is kept at or below 2,000 ohms. Under these circumstances there is no further change of the impedance during the time of current flow. When the current is broken, the impedance—to 20 microvolts—rises suddenly 80-150 ohms, depending on the "shock" tension applied; it then increases over a period of many seconds, approaching the original value asymptotically. The skin of human corpses reacts similarly to that of living individuals, but the impedance drop is less the longer the interval between the time of death and of examination.

REFERENCES

1. Gildemeister, M.: Die passiv-elektrischen Erscheinungen im Tier- und Pflanzenreich, Handb. d. norm. u. path. Physiol. 8: 657-702, 1928.
   Rein, H.: Die Elektrophysiologie der Haut. Handb. d. Haut- u. Geschlechtskr. 1: 1-160, 1929.
2. Jellinek, S.: Electrical Accidents From the Clinical and Forensic Standpoint, Proc. Roy. Soc. Med. 6: 17, 1913.
   Weeks, A. W.: Technical Investigation in Cases of Electrical Injury, Am. J. M. Jurisp. 2: 12, 1939.
3. Cerletti, U., and Bini, L.: Uno nuovo metodo di shockterapia, "l'elettroshock," Boll. Accad. Med. 136, 1938.
   Kalinowsky, L.: Electric-Convulsion Therapy in Schizophrenia, Lancet 2: 1232, 1939.
4. Spiegel, E. A.: Quantitative Determination of Convulsive Reactivity by Electric Stimulation of Brain With Skull Intact, J. Lab. & Clin. Med. 22: 1274, 1937.
5. Walter, W. G., and Fleming, G. W. T. H.: Electrically Induced Convulsions, Proc. Roy. Soc. Med. 33: 261, 1940.

SOME NEW ASPECTS OF MORPHINE ACTION: INFLUENCE OF PROSTIGMINE METHYLSULFATE ON EXCRETION*

DONALD SLAUGHTER, M.D., C. R. TREADWELL, PH.D., AND J. WESLEY GALES, M.D.
DALLAS, TEXAS

IT IS now well known (Gross and Thompson[1]) that the greater part of an injected dose of morphine sulfate can be recovered from the urine and feces of accustomed and nonaccustomed dogs. These authors pointed out, however, that a large part of excreted morphine was bound or conjugated and had to be hydrolyzed before it could be recovered.

Further, there is evidence[2] which indicates that prostigmine methylsulfate alters the accustoming† (tolerance-inducing) properties of morphine in dogs.

Recent work[3] suggests that prostigmine methylsulfate enhances the analgetic effects of morphine in cats. If this is true, the reason for the enhancement may lie in the action of prostigmine methylsulfate on the conjugation, destruction, and excretion of morphine.

For these reasons it seemed advisable to determine if the excretion of morphine was influenced by prostigmine methylsulfate. We were particularly interested in ascertaining whether prostigmine methylsulfate altered morphine excretion during withdrawal of morphine, comparatively, in accustomed and nonaccustomed animals and to note if the amounts of free or combined morphine were materially changed. In order to test such a hypothesis, both accustomed and nonaccustomed animals were used.

TECHNIQUE

The animals used in this study were all healthy female dogs. They varied in weight from 8 to 17.7 kg., but animals of similar weights were paired so that the difference in a pair (one accustomed and one nonaccustomed animal) was never greater than 2 kg. Five accustomed and five nonaccustomed animals were studied. A routine as outlined by Pierce and Plant[4] was followed in maintaining the dogs on a constant water and caloric intake. During a 6- to 7-day control period and during a 6 day excretion period, the normal body weights of these animals were not materially altered. The accustomed animals had previously been receiving 20 mg./kg. of morphine sulfate daily for 10 months or longer.

As mentioned above, one point of interest in this study was to compare the level of morphine excretion during withdrawal in accustomed and nonaccustomed

*From the Laboratories of Physiology and Pharmacology, and Biochemistry, Baylor University College of Medicine, Dallas, Texas.

A preliminary report was read at the Federated Society Meetings held in Chicago on April 15-19, 1941.

This work was aided by a grant to one of us (DS) from Hoffmann-La Roche, Inc., Nutley, New Jersey.

Received for publication, November 3, 1942.

†We prefer the term "accustomed" to the usual term "addicted," because in our opinion it reflects better the meaning of the French word "accoutumance."

dogs and to note what influence, if any, prostigmine methylsulfate had on the amount and form of morphine excreted. In order to adequately accomplish this, the following procedure was used: each animal, whether accustomed or nonaccustomed, received subcutaneous doses of 20 mg./kg. of morphine sulfate daily for an arbitrary three-day period. Then each animal was withdrawn for three days following the last dose. The morphine excretion over this period of six days constituted the control values. With these values were compared the amounts of morphine excreted after the same subcutaneous dose of morphine and over the same period but with the addition of prostigmine methylsulfate in subcutaneous doses of 0.2 mg./kg. administered in conjunction with the morphine as well as during the withdrawal period. The 24-hour output of feces was collected once daily. The animals were catheterized twice daily, and the urines were pooled, comprising the 24-hour output. The 24-hour sample of urine was analyzed individually for free and combined morphine, and the 24-hour sample of feces was analyzed for total morphine.

The extraction of free and of combined morphine after hydrolysis in the urine and the analysis of total fecal morphine was carried out as described by Gross and Thompson.[1] The morphine was determined colorimetrically by the diazotization procedure originally outlined by Pierce and Plant.[4] Known amounts of morphine were added to urine and feces. We were easily able to detect such known amounts and thus establish the validity of the method employed. Control diazotization experiments showed that prostigmine methylsulfate did not in any way alter the determination of morphine by this method.

RESULTS

Table I summarizes the effects of prostigmine methylsulfate on morphine excretion in accustomed and nonaccustomed animals. It is obvious that the total per cent of injected morphine excreted in urine and feces is reduced when prostigmine methylsulfate is administered with the morphine sulfate. This total difference is about the same in both groups of animals although there is a tendency for a greater disparity in the accustomed dogs. If the lessened excretion of morphine is broken down into component parts, the decreased urinary excretion in the nonaccustomed dogs is reflected almost two to one in the combined, as compared with the free form as shown in the following analysis.

ANALYSIS OF TABLE I. URINARY MORPHINE OUTPUT

Under prostigmine methylsulfate the total percentage reduction in the urinary morphine excretion in nonaccustomed dogs (NA) was 17.6 per cent; in accustomed dogs (A), 17.2 per cent; i.e., $\frac{67.2 - 55.4}{67.2} \times 100 = 17.6$ per cent and $\frac{69.1 - 57.2}{69.1} \times 100 = 17.2$ per cent.

From Table I we see that the actual percentage reduction of urinary morphine output under prostigmine is: For NA dogs, *free* 4.2 per cent; *comb.* 7.6 per cent; for A dogs, *free* 5.0 per cent; *comb.* 7.0 per cent. (Since fecal excretion represents only a small percentage of total excreted morphine, it is not considered in this analysis.)

The difference between the reduction in free and the reduction in combined morphine outputs was greater in NA dogs than in A dogs. Expressed as per cent of the reduction in free form for NA dogs, this difference was 81 per cent of the reduction of free form, while for A dogs it was 40 per cent of the reduction in free form; i.e., $\frac{7.6 - 4.2}{4.2} \times 100 = 81$ per cent, etc.

The percentage reduction of excreted morphine after prostigmine methylsulfate was for *Free Morphine* in NA dogs, 28 per cent; in A dogs, 34 per cent; for *Combined Morphine* in NA dogs, 14.6 per cent; in A dogs, 12.8 per cent; i.e., $\frac{15.0 - 10.8}{15.0} \times 100 = 28$ per cent, etc.

TABLE I

EFFECT OF PROSTIGMINE METHYLSULFATE ON MORPHINE EXCRETION IN NONACCUSTOMED (NA) AND ACCUSTOMED (A) DOGS

(Values Represent Average Per Cent Recovery of Total Dose of Morphine Administered)
(All Animals Received 20 Mg./Kg./Day of Morphine for 3 Days)

NUMBER OF ANIMALS		PROSTIGMINE METHYLSULFATE MG./KG./DAY		TOTAL URINE + FECES %		URINE						FECES %	
						TOTAL %		FREE %		COMBINED %			
NA	A	NA	A	NA	A	NA	A	NA	A	NA	A	NA	A
5	5	0.0	0.0	71.8	75.8	67.2	69.1	15.0	14.7	52.2	54.5	4.6	6.6
5	5	0.2	0.2	61.1	62.4	55.4	57.2	10.8	9.7	44.6	47.5	5.6	5.2

TABLE IA

OUTPUT OF FREE AND COMBINED MORPHINE IN TERMS OF TOTAL URINARY OUTPUT AS 100 PER CENT

"PROSTIGMINE"	TOTAL %		FREE %		COMBINED %	
	NA	A	NA	A	NA	A
0.0	100	100	21.3	21.2	77.7	78.8
0.2	100	100	19.5	17.0	80.5	83.0

The percentage reduction in amount of excreted, free morphine is slightly greater in A dogs than in NA dogs but is probably not significant. The percentage reduction in amount of excreted, *combined* morphine is slightly less in A dogs than in NA dogs, but the difference is also probably not significant.

If the figures are expressed as fractions of total urinary morphine found, instead of fractions of the administered morphine, the urinary figures of Table I are as follows: (See Table IA.)

Relative changes: Free morphine output reduction by prostigmine methylsulfate in NA vs. A dogs: NA dogs, 8.5 per cent; A dogs, 19.8 per cent. Hence on a basis proportional to the total morphine found, relative reduction of free morphine output by prostigmine methylsulfate is 2 to 3 times as great in A dogs as in NA dogs after prostigmine methylsulfate. There appears to be a relative *increase* of output of *combined morphine* after prostigmine methylsulfate: in NA dogs, 3.6 per cent; in A dogs, 5.3 per cent.

By way of explanation, the thought arises that perhaps prostigmine methylsulfate has less action on the excretory mechanism than it has on an earlier stage of morphine action and metabolism. That is, that the proportion of free

and combined morphine is an independent factor as far as prostigmine methylsulfate is concerned, and that any variation is more a function of the total amount excreted rather than total dose administered. When total excreted morphine of either accustomed or nonaccustomed animals is considered, we see that after prostigmine methylsulfate, 17 per cent less is excreted.

DISCUSSION

These results clearly indicate that prostigmine methylsulfate decreases the excretion of morphine in accustomed and nonaccustomed dogs. It seems fair to assume that such an effect demonstrates the ability of prostigmine methylsulfate to alter the potential effectiveness of morphine in the animal body. We have long felt that prostigmine methylsulfate prevented the development of tolerance in animals,[2] and clinical observations[7] using combinations of morphine and prostigmine methylsulfate suggest a similar effect.

The greatest actual decrease in excretion of morphine following prostigmine methylsulfate administration is reflected in the combined form. There is also, however, a reduction of output of the free form. In either case it seems possible to suggest that prostigmine methylsulfate alters the effectiveness of morphine in the body, either by causing retention of both forms or by increasing the rate of destruction of both forms in the body so that less can appear in the urine. In proportion to that amount of each form excreted without prostigmine methylsulfate, the greater proportional decrease of output is in the free morphine output; this is also evident in the relative shift in proportion of free and combined urinary morphine shown in Table IA.

Gross[6] has suggested that the combined form of morphine may be responsible for hypnosis and analgesia. If this is true and if retention of both forms of morphine in the body is the mechanism of prostigmine methylsulfate reduction of urinary output of morphine, one might thus explain, at least in part, the potentiation of morphine hypnosis and analgesia by prostigmine methylsulfate. The retention of free morphine might lead to a greater formation of combined morphine, which would still further potentiate the hypnotic action under Gross's hypothesis. Retention would result in a higher concentration of the effective form reaching the loci of action, and thus would simulate the effects of higher doses of morphine.

SUMMARY

1. Prostigmine methylsulfate in suitable doses decreases the total urinary excretion of morphine in accustomed and nonaccustomed dogs.

2. The bulk of the decreased morphine excretion is reflected in the combined form.

3. There is a relative shift in proportions of free and combined morphine in the urine, in the direction of a higher proportion of the combined form. This shift is greater in nonaccustomed dogs than in accustomed dogs.

4. In the light of a retention of morphine in the body produced by prostigmine methylsulfate, the mechanism of potentiation of morphine hypnosis and analgesia by prostigmine methylsulfate is discussed.

REFERENCES

1. Gross, E. G., and Thompson, V.: The Excretion of a Combined Form of Morphine in Tolerant and Non-Tolerant Dogs, J. Pharmacol. & Exper. Therap. 68: 413, 1940.
2. Slaughter, Donald: Some New Aspects of Morphine Action Tolerance and Addiction, ibid. 68: 302, 1940.
3. Slaughter, Donald, and Munsell. D. W.: Some New Aspects of Morphine Action Effects on Pain, ibid. 68: 104, 1940.
4. Pierce, I. H., and Plant, O. H.: Excretion of Morphine in Tolerant and Non-Tolerant Animals, ibid. 46: 201, 1932.
5. Slaughter, Donald, Parsons, J. C., and Munal, H. D.: New Clinical Aspects of the Analgesic Action of Morphine, J. A. M. A. 115: 2058. 1940.
6. Gross, E. G.: Personal Communication.

IMMUNOLOGIC AND TOXIC PROPERTIES OF CASEIN DIGEST AS PREPARED FOR PARENTERAL ADMINISTRATION*

HOWARD C. HOPPS, M.D., AND JAMES A. CAMPBELL, B.S., CHICAGO, ILL.

ALTHOUGH hypovitaminosis continues to receive preponderant emphasis in studies on malnutrition, the effects of protein deficiency are not to be minimized. In addition to such obvious effects as inanition, failure of growth, anemia and nutritional edema, protein deficiency (hypoproteinemia) affects both quantitatively and qualitatively the regeneration of tissue; it predisposes the liver to injury by toxins, and it seriously interferes with the synthesis of antibody globulin. Hypoproteinemia is an important cause of delayed wound healing and wound dehiscence.[1] An increased susceptibility of the liver to injury by such toxic substances as chloroform and arsphenamine during a state of protein depletion has been shown, and the fact that feeding of protein exerts a marked protective action against injury has been demonstrated.[2] The effect of protein depletion on resistance to infectious disease was the subject of discussion in the presidential address to the American Association of Immunologists this year.[3] Evidence was presented to show that hypoproteinemia in animals markedly decreased their ability to synthesize antibody globulin, thus interfering with one of their most important defense mechanisms against infectious disease —the ability to develop active immunity.

The treatment or prevention of the protein deficient state is often difficult. Administration of protein by mouth may be impossible, or digestive disturbances may prevent assimilation of fed protein. Associated with these conditions there is often a marked increase in protein requirements either from abnormal loss of protein or from increased metabolism. Parenteral therapy to relieve a condition of protein deficiency would be of especial importance in preparation of patients for surgery as well as in helping to maintain their nutritional state during the early postoperative period. In a discussion of hypoproteinemia and its relation to surgical problems, Ravdin[4] states, "No consideration of fluid and electrolyte loss and their restitution is sufficient unless the plasma protein is simultaneously considered."

*From the Department of Pathology, University of Chicago, Chicago.

Received for publication, November 4, 1942.

For these reasons a source of protein which may be safely administered parenterally is much to be desired, to supplement or complement solutions of carbohydrates, salts, and vitamins.

Over thirty years ago experiments were first done to develop such a source of protein. Success has come only recently, however. The gradual accumulation of facts leading to the demonstration that protein requirements could be completely or almost completely met by supplying ten "essential" amino acids was probably the most influential factor in this respect. In a Harvey lecture in 1934[5] Rose said, "Indeed it is not wholly improbable that methods may be developed whereby this ideal mixture (amino acids) may be employed for parenteral administration to human subjects when the prevention of undue loss of body structure is an important consideration."

This "ideal mixture" of amino acids has been closely approached in recent years although it comes not from a synthetic mixture of purified amino acids as Rose had postulated, but from the hydrolysis of a complete protein. Rapidly accumulating experimental data[6, 7, 8, 11, 13] indicate that such preparations are utilized for protein synthesis and that when given parenterally, as a sole source of nitrogen, they serve to maintain nitrogen equilibrium and support normal growth and tissue repair.

A commercial preparation of casein hydrolysate designed to serve as a parenteral source of protein food has been available for experimental use and has received much study during the past five years.* Experience with new drugs (e.g. amidopyrine, dinitrophenol, sulfanilamide, etc.), has indicated, however, that although initial experimental trials reveal no significant toxic action, there may be decided deleterious effects when such drugs are subject to widespread or general use. For this reason, new drugs should always be subjected to thorough experimental study expressly designed to detect or to determine the nature of possible toxic effects. The demonstration of such toxic or deleterious action of specific drugs need not result in absolute contradiction to their use; yet the knowledge of such untoward effects may allow for intelligent, directed caution or for the application of corrective measures. With these thoughts in mind experiments were undertaken to determine whether or not amino acid solutions prepared from hydrolyzed casein were toxic.

In preparations made from proteins and intended for intravenous administration to human beings the possibility of allergic reaction must always be considered. Since casein is an antigenic protein, there is the possibility that a product derived from casein might retain some of these antigenic properties. The first consideration, therefore, is to determine whether or not casein hydrolysate is antigenic. Since it is generally accepted that complete hydrolysis destroys the biologic speciation of native proteins, demonstration of nonantigenicity in these products would be strong evidence that complete hydrolysis had been accomplished.

Two commercial preparations of casein hydrolysate were selected for testing. These will be referred to as products A and B. Concerning product A, its manufacturer states that efforts have been made to sensitize guinea pigs with A and to shock milk-sensitive pigs with A solution and that all such studies have been negative, indicating the absence of antigenic properties; however, no

*Amigen, an enzymatic hydrolysate of casein, prepared by Mead Johnson & Co.

details of these experiments are available in the literature, nor are there experiments specifically directed toward the detection of pork protein which might have been introduced during the course of hydrolysis, a process accomplished by the action of ground pork pancreas. Concerning the antigenic properties of product B, an acid hydrolysate of casein, its manufacturer states that "biologic speciation in most instances is due to the prosthetic groups attached to the protein. Most proteins shown to be antigenic substances are despeciated by enzymatic hydrolysis." The product in question, however, is not an enzymatic hydrolysate. The works of Hill and of Altshuler are cited to substantiate further claims that this particular casein digest is nonantigenic. Neither Hill nor Altshuler used the particular preparation under consideration. Hill[7] maintained infants allergic to milk on casein digest for periods up to three months. In about half of the cases eczema was relieved. Altshuler, Hensel, and Sahyun[8] injected two rabbits with an acid hydrolysate of casein and were subsequently unable to produce anaphylactic shock in these animals. These findings are inconclusive, however, because of the unsuitability of rabbits for anaphylactic tests.

EXPERIMENTAL

ANTIGENIC PROPERTIES OF CASEIN HYDROLYSATES

The first series of experiments consists of determining whether or not preformed antibodies to casein react with hydrolysates of casein and whether or not hydrolysates of casein are in themselves capable of stimulating the formation of antibodies for themselves or for casein. Three groups of normal adult rabbits were given repeated injections of preparation A,* preparation B, and purified casein (Casein-Harris). Multiple doses of these substances were administered by intramuscular, intraperitoneal, subcutaneous, and intravenous routes. In addition, ten intramuscular "depots" of the test substance adsorbed upon alumina gel after the method of Hektoen and Welker[9] were produced. After 65 days serum precipitin titers were determined in all groups by the collodion particle method of Cannon and Marshall,[10] as well as by the usual antigen-dilution method. Precipitins for casein digest were not found in any case; neither did the animals treated with casein digest exhibit antibodies for casein. Skin tests were performed using cows' milk, 10 per cent A, and 10 per cent B. Casein-sensitized animals gave positive reactions only to milk. Animals treated with casein digests gave no positive reactions to casein nor to the casein digest with which they had been treated. These animals were bled, and the pooled serums of each group were injected intraperitoneally into corresponding groups of normal 180- to 200-gram guinea pigs. Two days later anaphylactic tests were performed. Two c.c. portions of the test substances were given via the jugular vein to the unanesthetized pigs. Casein-sensitized animals were divided into three groups. To group one, casein was given and these animals died of typical anaphylactic shock. Group two received A (10 per cent solution) with no reaction. After several hours these same animals were again injected, this time with casein, with resultant death from typical anaphylactic shock. Group three was treated in essentially the same manner as group two, except that solution B was substituted for A. Identical results were obtained.

*Kindly furnished by Mead Johnson & Co.

Twenty-eight virgin female guinea pigs weighing 150 to 180 grams were "passively sensitized" with pooled serums from rabbits treated with casein, A, or B. These animals were sacrificed at three to four days, and muscle strips (uterine horn) preparations were studied for passive sensitization using the Schultz-Dale technique. Fig. 1 shows the typical reaction of a normal uterine muscle strip to A, to B, and to casein. These test solutions (aqueous) had been adjusted to a pH of 6.8 to 7.0 by the careful addition of 0.1 N NaOH. In Fig. 2, however, the pH of test solutions A and B was unaltered (approximately 4.5). These differences in reaction to A (compare Figs. 1 and 2) are apparently the result of some change in state that occurs with neutralization of solution A, a change that is marked by the gradual formation of a rather abundant cloudy precipitate.

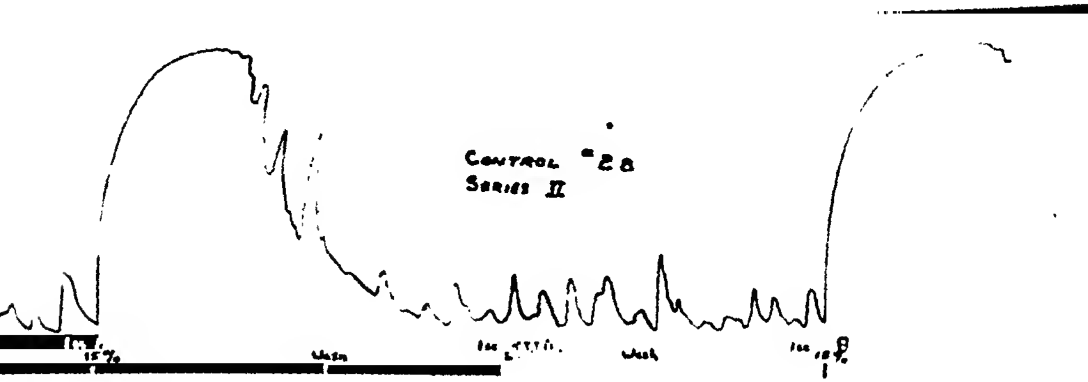


Fig. 1.

Fig. 2.

The reaction of normal smooth muscle to these hydrolysates of casein indicates the presence of some histamine-like substances, peptones, or tyramine, which may explain the frequently observed reactions of flushing, sensation of warmth, and nausea observed in patients who are administered casein digest by vein.[11, 13]

Typical reactions of the casein-sensitized group are shown in Fig. 3. An anaphylactic response has occurred to casein. The reactions of this group to solutions A and B are essentially similar to those exhibited by the control animals.

Fig. 4*A* shows a typical reaction of the A-"sensitized" group to casein and to A; these are normal reactions. Fig. 4*B* illustrates the reaction of a B-"sensitized" animal to casein and to B. This reaction to B is the most marked of any exhibited by this group; it is not a typical anaphylactic reaction, however, and the reaction to casein is as in the normal. Other animals of this group gave an essentially "normal" reaction to B.

These studies adequately demonstrate that the two preparations of hydrolyzed casein tested are incapable of stimulating antibody production, nor do they react to preformed casein antibodies.

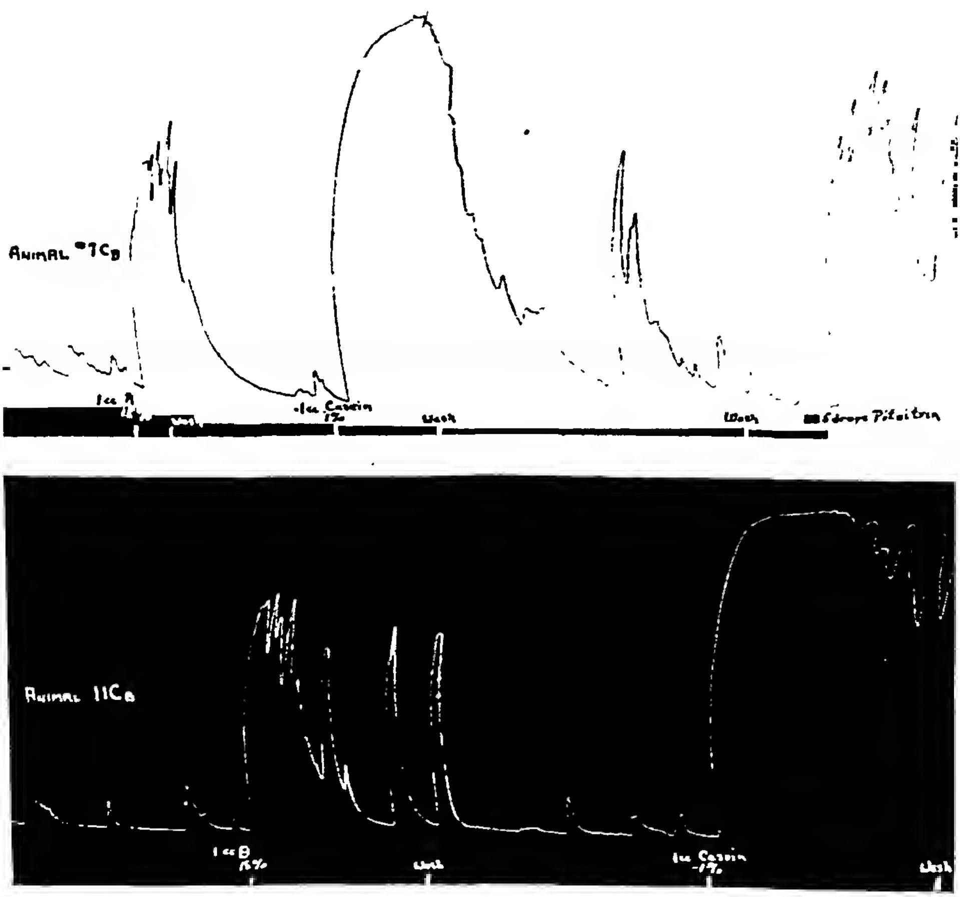


Fig. 3.

A second series of experiments concerns only product A in the preparation of which hydrolysis is accomplished by means of ground pork pancreas. The nonantigenicity of substance A has just been demonstrated, but there remains for investigation the possibility that modified pork protein exists as a partial antigen or haptene, incapable of stimulating antibody formation, but capable of reacting with preformed pork antibodies. To explore this possibility, rabbits were sensitized to swine pancreas and swine serum, as demonstrated by the presence of specific precipitins and by positive Arthus reactions. Animals treated with A failed to show precipitins or positive skin reactions to swine

serum or swine pancreas. Likewise, animals sensitized with swine pancreas or swine serum failed to show precipitins or positive skin tests against A. Anaphylactic tests were performed with passively sensitized guinea pigs in the same manner as previously described. There was no demonstrable antigenic relationship between pork proteins and product A.

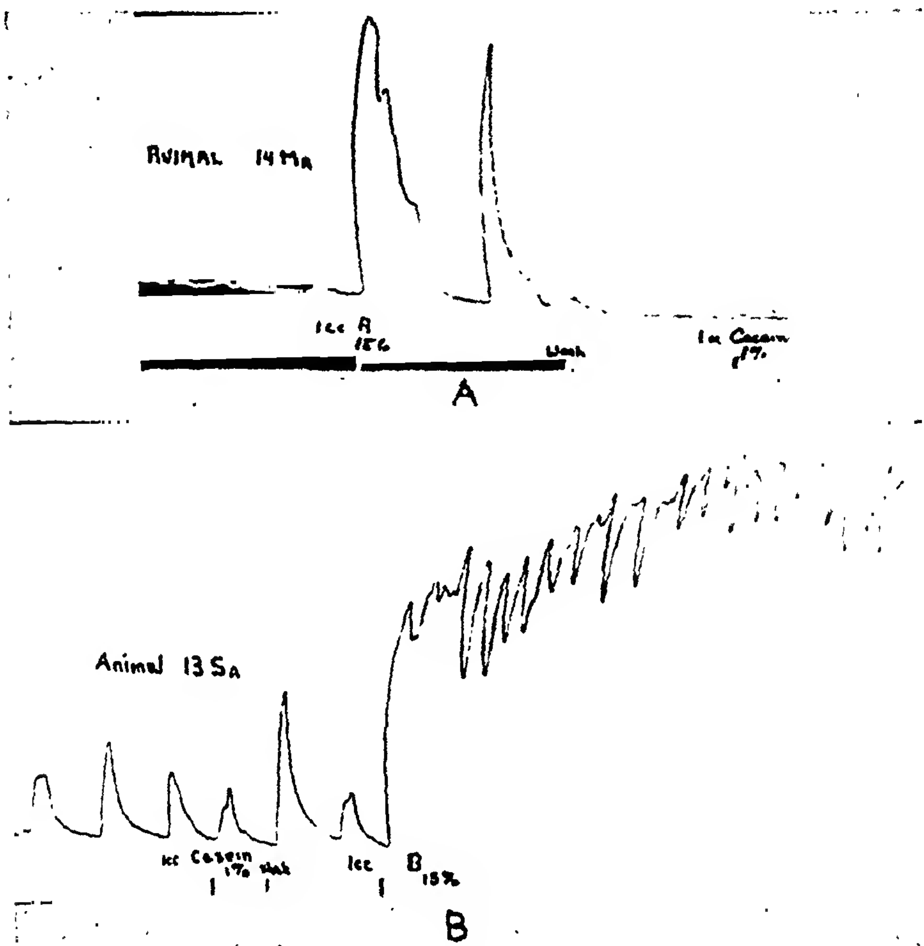


Fig. 4.

TOXIC PROPERTIES OF CASEIN HYDROLYSATES

Table I shows the hydrogen ion activity and hydrogen ion concentration of the two preparations tested. It can be seen that these solutions have a hydrogen ion concentration comparable to 1/16 to 1/12 N HCl. Their effect on the pH and CO_2 combining power of human plasma in vitro is also shown. The proportions of hydrolysate and plasma in these mixtures is comparable to that which would be attained in a 65 kg. patient given intravenously, at a single dose, 1500 c.c. of a 10 per cent solution of amino acid mixture. This is admittedly an artificial comparison in that amino acid mixture introduced into a patient would not all remain in the blood stream for the period required to administer this large quantity. From these observations it was thought that

casein hydrolysates might have a marked tendency to produce acidosis, especially in those patients who were seriously ill and who received repeated large doses of the substance. Experiments were done to evaluate this effect. Blood plasma was obtained from two patients* just before the intravenous administration of 1500 c.c. of 10 per cent amino acids (product A). This material was given over a period of eight hours and was accompanied by 1500 c.c. of 10 per cent dextrose in saline and 1500 c.c. of 10 per cent dextrose in water. Immediately upon completion of this infusion a second specimen of plasma was obtained from each patient. In patient No. 1, the CO_2 combining power dropped from 59.2 vol. per cent in the first sample to 50.5 in the second sample. In patient No. 2, the decrease was from 58.7 vol. per cent to 52.6 vol. per cent. These changes may well represent only the dilution of blood plasma resulting from administration of a large amount of hypertonic fluid. Plasma pH did not vary significantly. Similar observations were made on a third patient† to

TABLE I

HYDROGEN ION ACTIVITY AND CONCENTRATION OF CASEIN HYDROLYSATES AND THEIR EFFECT ON HUMAN PLASMA

SOLUTION	pH	0.1 N.Na OH ADDED TO BRING pH TO: (10 C.C. SAMPLE) 7.2—7.4	8.1—8.2
10% A	4.6	5.59 c.c.	12.82 c.c.
10% B	4.33	8.15 c.c.	13.98 c.c.
		CO_2 Combining Power Vol. %	
Plasma + Saline	7.5	60.9	
Plasma + A	5.96	6.9	
Plasma + B	5.51	5.8	

whom 400 c.c. of 10 per cent amino acids was given intravenously during a period of two hours and thirty minutes. Variation in CO_2 combining power and pH of plasma did not exceed the limits of experimental error.

From these in vivo studies it is apparent that hydrolyzed casein, in spite of its very appreciable hydrogen ion activity in vitro, has but little effect on the acid-base balance in the body. Since but a small proportion of this substance, given intravenously, is excreted as such in the urine, and since there is but little immediate destruction of the substance, as measured by increased ammonia excretion, it appears necessary to postulate the very rapid conjugation of parenterally administered amino acids in order to explain how the acidity of such solutions is nullified without affecting the alkaline reserve of the blood. That such conjugation of amino acids results in the immediate formation of complete proteins seems hardly likely although such a step may be a major one in the synthesis of protein. This conclusion is in harmony with direct observation on the rapidity with which the blood stream is cleared of injected amino acids.[12, 13]

To further test for toxic action of casein hydrolysate, post-mortem studies were made on seventeen patients who had received casein digest by intravenous route during the course of their terminal disease. Of these patients, the maximum

*These individuals were both adult women. Patient No. 1 had undergone a cholecystectomy, and patient No. 2, an appendectomy, the preceding day. They both made uneventful recoveries. These patients were on the service of Dr. Alexander Brunschwig.

†A 56-year-old white man suffering from lipoid glomerular-nephritis (nephrotic nephritis).

amount administered was 2,100 Gm., as 10 per cent solution (over a 21-day period). Time of administration of amino acids in these patients varied from twenty days to one hour before death. In no case were there morphologic changes in tissues which could be definitely attributed to a toxic effect of casein hydrolysate. In but one instance was there any reason to suspect that amino acid therapy was a factor in causing death. This was in a man with carcinoma of the cystic bile duct who had undergone an operation in which the gall bladder and cystic duct were resected, the common bile duct ligated, and the hepatic artery ligated and severed. Postoperatively, this patient's course was one of progressive failure. There was jaundice and, two days before death, a precipitous fall in serum protein. In an attempt to ameliorate this condition amino acid therapy was instituted. Following the intravenous administration of 200 c.c. of 10 per cent amino acid solution, the patient experienced a severe chill. The venous infusion was stopped, but the patient died an hour later. At necropsy severe hepatic damage was evident.

That the liver is the major organ concerned with the synthesis of protein seems evident from the work of Whipple, Mann, and others; in fact, the disappearance curve for injected amino acids has been used with some success in evaluating liver function. The study of intermediary protein metabolism with the aid of isotopes by Schoenheimer and others also lends support to the view that amino acids, plasma proteins, and tissues proteins, especially those of the liver, exist in a state of very dynamic equilibrium. In a recent paper,[14] Madden and Whipple summarize such experimental evidence and conclude, "The present evidence speaks in favor of the liver as the site of production of albumin and much of the globulin."

The case last cited may well represent a situation in which hepatic damage was so severe that rapid conjugation of amino acids, as a preliminary step in the synthesis of protein, could not occur, with the result that amino acids given intravenously exerted the same effect on the acid-base balance of the body as was observed in in vitro experiments with human plasma (see Table I). It suggests that marked hepatic insufficiency may be a very serious contraindication to the parenteral administration of amino acid mixtures.

SUMMARY AND CONCLUSIONS

It has been demonstrated that the two preparations of casein hydrolysate tested are nonantigenic. Reactions of normal smooth muscle strips to casein digests have indicated the presence of some histamine-like substances, peptones, or tyramine, which may explain the frequently observed reactions of flushing, sensation of warmth, and nausea, in patients administered casein hydrolysate by vein. It has been shown that although casein hydrolysates have a high hydrogen ion concentration and activity, this does not appreciably affect the acid-base balance in those persons with adequate liver function. This is presumably due to the very rapid rate at which amino acids are conjugated by the liver. Post-mortem studies of seventeen individuals who had received casein digest parenterally failed to disclose significant morphologic changes as a toxic manifestation of amino acid therapy. The suggestion is made that patients with poor liver function may be unable to conjugate parenterally administered amino acids at a sufficiently rapid rate to escape severe acidosis.

REFERENCES

1. Rhoads, J. E., Fliegelman, M. T., and Panzer, L. M.: The Mechanism of Delayed Wound Healing in the Presence of Hypoproteinemia, J. A. M. A. 118: 21, 1942.
2. Goldschmidt, S., Vars, H. M., and Ravdin, I. S.: The Influence of the Foodstuffs Upon the Susceptibility of the Liver to Injury by Chloroform, and the Probable Mechanism of Their Action, J. Clin. Investigation 18: 277, 1939.
Messinger, W. J., and Hawkins, W. B.: Arsphenamine Liver Injury Modified by Diet. Protein and Carbohydrate Protective, but Fat Injurious, Am. J. M. Sc. 199: 216, 1940.
Miller, L. L., and Whipple, G. H.: Chloroform Liver Injury Increases as Protein Stores Decrease, Am. J. M. Sc. 199: 204, 1940.
3. Cannon, P. R.: Antibodies and the Protein-Reserves, J. Immunol. 44: 107, 1942.
4. Ravdin, I. S.: Hypoproteinemia and Its Relation to Surgical Problems, Ann. Surg. 112: 576, 1940.
5. Rose, W. C.: The Significance of the Amino Acids in Nutrition, The Harvey Lectures, 1934, p. 49.
6. Elman, R.: Intravenous Injection of Aminoacids in Regeneration of Serum Protein Following Severe Experimental Hemorrhage, Proc. Soc. Exper. Biol. & Med. 36: 867, 1937.
Elman, R.: Urinary Output of Nitrogen as Influenced by Intravenous Injection of a Mixture of Amino-Acids, Ibid. 37: 610, 1937.
Elman, R.: Parenteral Replacement of Protein with the Amino-Acids of Hydrolized Casein, Ann. Surg. 112: 594, 1940.
Mueller, A. J., Kemmerer, K. S., Cox, W. M., Jr., and Barnes, S. T.: Effect of Casein and a Casein Digest on Growth and Serum Protein Regeneration, J. Biol. Chem. 134: 573, 1940.
7. Hill, L. W.: Amino Acids as a Source of Nitrogen for Allergic Infants, J. A. M. A. 116: 2135, 1941.
8. Altshuler, S. S., Hensel, H. M., and Sahyun, M.: Maintenance of Nitrogen Equilibrium of Amino Acids Administered Parenterally, Am. J. M. Sc. 200: 239, 1940.
9. Hektoen, L., and Welker, W. H.: Precipitin Production in Rabbits Following Intramuscular Injection of Antigen Absorbed by Aluminum Hydroxide, J. Infect. Dis. 53: 309, 1933.
10. Cannon, P. R., and Marshall, C. E.: An Improved Serologic Method for the Determination of the Precipitative Titres of Antisera, J. Immunol. 38: 365, 1940.
11. Shohl, A. T., Butler, A. M., Blackfan, K. D., and MacLachlan, E.: Nitrogen Metabolism During the Oral and Parenteral Administration of the Amino Acids of Hydrolyzed Casein, J. Pediat. 15: 469, 1939.
Farr, L. E., and Mac Fadyn, D. A.: Amino-Acid Nitrogen in Urine of Children with the Nephrotic Syndrome Following Intravenous Amino Acids, Proc. Soc. Exper. Biol. & Med. 42: 444, 1939.
Farr, L. E.: The Intravenous Administration of Small Doses of Casein Hydrolysate to Nephrotic Children and Its Effect Upon the Nitrogen Balance and Plasma Amino Acid Level, J. Pediat. 16: 679, 1940.
Farr, L. E., Emerson, K., Jr., and Futcher, P. H.: The Comparative Nutritive Efficiency of Intravenous Amino Acids and Dietary Protein in Children with the Nephrotic Syndrome, Ibid. 17: 595, 1940.
12. Elman, R.: Amino-Acid Content of the Blood Following Intravenous Injection of Hydrolyzed Casein, Proc. Soc. Exper. Biol. & Med. 37: 437, 1937.
13. Landesman, R.: and Weinstein, V. A.: The Intravenous Use of Amino Acids for Nutritional Purposes in the Surgical Patient, Surg., Gynec. & Obst. 75: 300, 1942.
14. Madden, S. C., and Whipple, G. H.: Plasma Proteins: Their Source, Production, and Utilization, Physiol. Rev. 20: 194, 1940.

CAFFEINE-WITHDRAWAL HEADACHE*

Robert H. Dreisbach, Ph.D., and Carl Pfeiffer,† Ph.D., M.D., Chicago, Ill.

THE production of the experimental headache for investigative purposes was initiated by the observations of Loevenhart[2] and his co-workers in 1927, who found that nitrite headaches were accompanied by a dilatation of the meningeal blood vessels. Pickering[3] in 1933 studied headaches produced by the intravenous injection of small amounts of histamine. These studies were enlarged by Clark et al.[4] in 1934 and by Wolff et al.[5] in 1940, who demonstrated that this experimental headache was accompanied by an increased excursion of the cerebral blood vessels. Carbon monoxide headaches have been studied in a few individuals (Cobb et al., 1924),[6] but the toxic nature of carbon monoxide does not lend itself to human experimentation. The experimental headaches studied thus far do not simulate any of the so-called common headaches in mode of onset or duration. The histamine headache, however, is by far the most constant and may be produced in almost all subjects.

Because many people ascribe an occasional headache to lack of their morning coffee, and because considerable "letdown" must result if this stimulant drug is withdrawn from habituated individuals, an attempt to produce and study such a headache was undertaken. In a recent survey of one hundred and twenty-eight migraine patients, twenty-five stated that lack of their usual coffee intake would result in headache. Five patients volunteered the information that the headache was not of the migraine type. In this study we have attempted to produce headache by administration of caffeine over a suitable period, usually a week, and then abruptly withdrawing the drug.

Data obtained in this laboratory[7] have indicated that there is a relative hemoconcentration accompanying migraine. Hence, it was thought advisable to determine total proteins and hematocrits during the course of the experiments. Several investigators[8-11] have reported that the serum calcium is decreased in migraine patients. Therefore, it was thought possible that electrolyte changes in the blood might also be correlated with any period of headache produced by caffeine withdrawal.

EXPERIMENTAL

Caffeine, 0.120 Gm. capsules (gr. ii) as the sodium benzoate or citrate salt, was administered to normal subjects in an increasing daily dosage up to 0.78 Gm. (gr. xii), usually given in the morning. Twenty male and two female subjects (graduate and medical students) were used. Their ages varied from

*From the Departments of Pharmacology of the University of Chicago and Wayne University Medical Schools.

A preliminary report was given before the American Society for Pharmacology and Experimental Therapeutics at New Orleans, March, 1940.

Aided in part by a grant from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

†Lieutenant (M.C.), U.S.N.R.

Received for publication, November 16, 1942.

20 to 33 years. They were *not* hospitalized, but they were observed and had their symptoms recorded daily. No regulation of food intake was attempted. Only three of the twenty subjects were normally total abstainers from caffeine-containing beverages. In order to add to our knowledge of the migraine syndrome, five of the subjects were particularly chosen because they had had typical periodic migraine headaches for at least two years. The subjects were given a series of ten or more dated envelopes, each containing the same number of capsules, placebo capsules and caffeine capsules being mixed in such a way that the subject would start with a small dose of caffeine the first day, but increasing daily so that by the sixth or seventh day the subject would be receiving 10 to 12 grains of caffeine per day. On the seventh or eighth day placebo capsules were substituted without the subject's knowledge, so that no caffeine was received thereafter. During the experiment the subject abstained from caffeine beverages, and in most cases the subjects had no caffeine beverages for the 24 to 48 hours preceding the beginning of administration. The experiment was postponed if the subject had a headache, and this occurred in five instances when habituated subjects discontinued their customary daily coffee intake.

Five blood samples were taken during each experiment. An initial control sample was taken before administration of caffeine was started, a second at the height of the caffeine stimulation, a third on the day before withdrawal, a fourth at the height of the headache, and a fifth on the following day when the subject was free of headache. In tabulating the results, the first three samples on each subject have been averaged, since they were found to be uniformly the same. At the time the blood samples were taken, blood pressure and pulse were recorded after the subject had been supine for five minutes. All control observations and samples were taken between 4 P.M. and 8 P.M. after it was found that this was usually the time of maximum headache.

Serum calcium, serum inorganic phosphorus, serum potassium, serum protein, serum and blood specific gravity, and hematocrits were determined as in previous studies.[12] The calcium samples were stored at a stable phase of the analysis, so that fifty to one hundred samples could be disinterestedly titrated.

RESULTS

During the first two or three days of caffeine administration, all subjects reported stimulation with slight muscular tremors and a lessened sense of fatigue. Two subjects noted insomnia immediately upon retiring, but this was never severe enough to require sedation. Later they noted no stimulation, but did report that they slept longer and more soundly. Approximately one-half of the subjects reported diuresis during the early period of the administration. The lack of stimulation on repeated doses agrees with the findings of Eddy and Downs,[13] who noted an increase in the reaction time in habituated subjects.

In twenty-one out of thirty-eight trials, headache resulted which was as severe as the subject had ever had. In eleven trials the headache was definite, but not severe enough to necessitate treatment. In six experiments on four subjects very slight or no headache resulted. The headache-resistant group did not include any of the original abstainers from caffeine. In three of these re-

sistant subjects, the icteric index was above the normal range[23, 11, 8] and the sclerae of M. B. with an index of 23 were yellow-tinged. These three subjects have noted that they are generally free of headache of any type. However, subject M. B. with idiopathic jaundice had a typical histamine headache of 12 minutes' duration when given 0.1 mg. of histamine diphosphate intravenously. Both of the two female subjects developed severe headache which required treatment.

Characteristics of the Headache: The symptomatology of the headache is remarkably constant and may be summarized as follows: On the day of withdrawal, lethargy was usually noticeable in the morning, while a feeling of cerebral fullness occurred about noon. Actual headache usually began in the early afternoon and reached a peak three to six hours later. The subjects localized the headache as occipital or central at the onset, which then in most cases became generalized and throbbing in character. In six subjects the headache was consistently accompanied by some degree of nausea, and in two subjects vomiting occurred. In four subjects the headache was consistently accompanied by a serous rhinorrhea of such severity that they at first thought that an acute upper respiratory infection was impending. Subjects also complained of mental depression, drowsiness, yawning, and disinclination to work.

During the incipient stage of the headache the patients' ears were usually intensely hyperemic, but later if the patients became nauseated, the ears would pale and generalized pilomotor activity was observed. The headache, when well established, was intensified by bending over or by straining, as at stool (Valsalva's experiment). Relaxation, such as napping, made the headache worse, while violent physical exercise (running upstairs), with the exception of one subject, intensified the headache. Jugular vein compression with the resultant increase in cerebrospinal fluid pressure produced the usual feeling of cerebral fullness, but did not intensify the headache.

Treatment of the Headache: Headache produced by caffeine withdrawal was found to respond to treatment by caffeine or acetylsalicylic acid. When caffeine was used to relieve the headache, the subjects sometimes complained of secondary headache the next day. While benzedrine sulfate gave relief for two to six hours, in four of six subjects the headache recurred in an intensified form. Amyl nitrite afforded temporary relief in one subject, but this was then followed by a short period of exacerbation, after which the headache continued as before. In severe headache with nausea and vomiting, oxygen inhalation afforded definite relief in two subjects. If given early enough in the course of the headache, the most effective treatment was caffeine.

Blood Findings: In Table I are given the results of determinations of serum calcium, serum inorganic phosphorus, serum protein, and hematocrit during the normal and administration periods, at the time of the headache, and on the day following when the subjects were free of headache or had received caffeine in treatment of a secondary headache. Under each column are given the averages and standard deviations of all the data. Values obtained during the administration of caffeine are not tabulated separately because these values were comparable to the normal values. The serum protein and the hematocrit were found to be generally lower at the time of the headache in all but three

experiments. The serum calcium was lower in all but one experiment, and serum inorganic phosphorus was slightly higher during the headache period. The change in serum calcium could be ascribed to the hemodilution except that by the same reasoning the rise in serum phosphorus would be even greater than is apparent from the data. Since calcium fluctuates inversely with phosphorus, the calcium may still be indirectly involved.

Graphs of changes in blood constituents in one subject during three different experiments are shown in Figs. 1 to 3. It should be noted that at the time of the headache, significant, but not consistent, decreases occurred in the serum calcium, protein, hematocrit, and blood specific gravity. An elevated serum phosphorus was consistently present at the time of the headache. The blood pressure and pulse changes were frequently marked, but were in general uninterpretable.

TABLE I

SUBJECT		CALCIUM MG. PER 100 C.C.			PHOS. MG. PER 100 C.C.			PROT. GM. PER 100 C.C.			HEMATOCRIT %		
		1	2	3	1	2	3	1	2	3	1	2	3
D	10/7	9.6	9.5	10.0	3.2	4.6	4.2	8.2	7.6	8.3			
P	10/17	9.6	9.2		4.3	4.2	3.0	7.7	8.1	7.6	47	48	49
R	10/27	9.8	9.4	9.5	2.6	3.6	3.0	8.1	7.8	8.3	48	45	48
KA	11/10	9.5	9.2	9.0	3.0	3.2	3.1	8.8	8.5	8.2	49	48	49
RS	11/10	9.0	8.1	8.2	2.7	3.4	3.2	8.0	7.5	7.5	47	46	49
BF	12/9	9.6	9.0	8.1	3.5	3.8	4.1	8.3	8.0	7.9	47	44	46
D	12/1	9.6	9.5	10.0	2.9	3.2	3.4	8.3	8.7	8.9	47	46	51
D	12/29	10.3	9.3	10.9	3.6	3.1	3.4	8.8	7.7	8.8	51	48	49
R	1/19	9.9	9.5	9.8	2.6	2.9	2.7	8.7	8.4	8.7	48	45	47
P	2/23	9.3	8.9	9.5	3.0	3.2	3.6	8.0	7.1	8.7	50	46	48
D	2/23	9.4	9.1	8.9	3.6	3.6	2.8	8.2	7.8	7.6	52	48	50
BF	4/6	9.2	9.0	8.6	3.4	3.3	3.4	7.6	7.4	7.2	43	45	44
DH	4/15	9.9	9.5	9.7	2.8	2.8	3.7	8.1	7.9	8.3	50	49	51
RS	4/23	8.9	8.9	8.9	3.6	3.8	3.0	8.2	7.7	7.8	47	46	43
RS	6/14	9.2	8.5	9.1	4.3	4.7	3.6	8.4	8.4	7.0	48	45	49
DB	5/6	9.6	9.3	10.8	3.5	3.6	3.7	8.0	7.9	9.6	49	48	47
CL	6/29	9.0	8.8	8.8	2.6	3.5	4.1	7.2	7.5	7.4	48	51	47
Average		9.5	9.1	9.4	3.2	3.6	3.4	8.2	7.9	8.1	48.2	46.7	47.9
Median		9.6	9.2	9.3	3.2	3.5	3.4	8.2	7.8	8.2	48	46	48
S.D.		±.4	±.4	±.8	±.5	±.5	±.4	±.4	±.4	±.5	±2	±2	±2

The first column in each series represents the average of all determinations taken before the headache. Column 2 represents determinations at the time of the headache, and column 3 represents determinations 24 hours after the headache. The mean of 12 control calcium samples taken daily at 5 P.M. on 4 subjects for 3 days was 9.45 ± .57 mg. per cent.

DISCUSSION

It was uniformly noted that the headache following caffeine withdrawal was quite different from the migraine syndrome in the five migraine subjects studied, although the caffeine-withdrawal headache was accompanied by nausea in four of the migraine subjects and vomiting in one. In two subjects whose migraine is typically unilateral the caffeine-withdrawal headache was generalized. In two subjects the headache occurred on the opposite side of the head from their usual migraine. One subject has left-sided frontal migraine, but three caffeine-withdrawal headaches were bilateral and occipital in distribution. Three of these five subjects regularly have scotomas with their migraine headaches, but no such visual phenomena preceded or accompanied this experimental headache. Two migraine patients, however, had migraine headaches at the height

of the stimulant action of caffeine, and in one of these subjects this again occurred when another caffeine-withdrawal headache was attempted. It would thus appear that the true migraine headache is apt to occur during the period

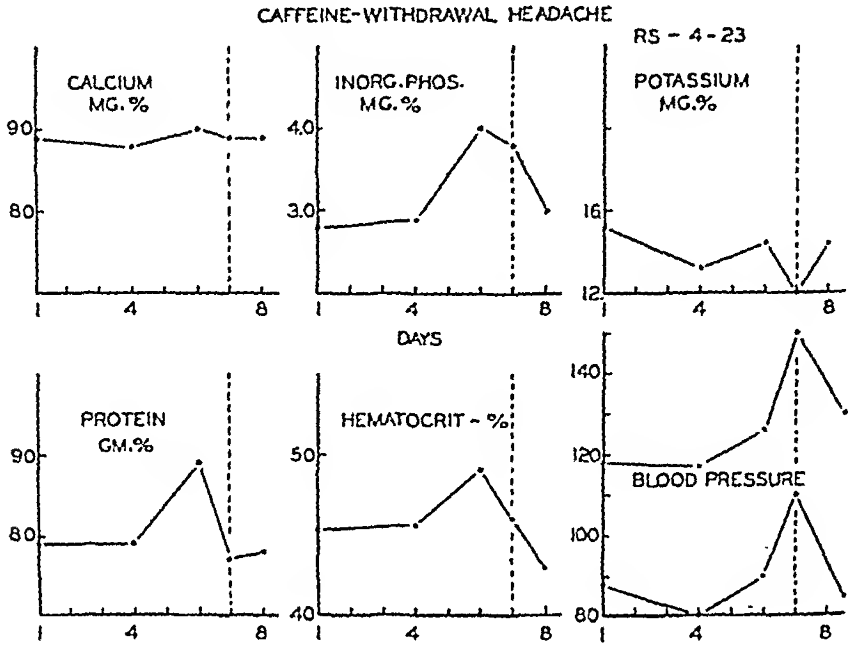


Fig. 1.

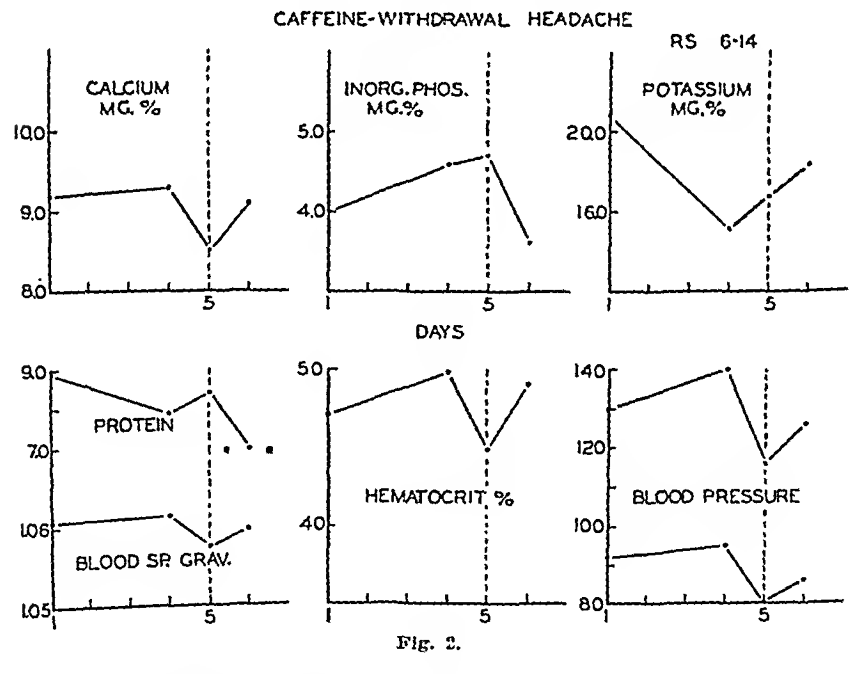


Fig. 2.

of caffeine overstimulation,* while the withdrawal of caffeine results in a distinct type of generalized headache. In a recent study[7] of migraine headache, we found no consistent changes in the serum calcium or phosphorus. A slight

*This led to an erroneous statement in the original Proceedings report.[1]

rise in hematocrit and total proteins was present at the time of the headache. The change in blood chemistry and blood volume found in this study would, hence, be another point of dissimilarity between the two types of headache.

That the caffeine-withdrawal headache is due to changes in cerebrospinal fluid pressure or volume is doubtful, since bilateral jugular compression which raises cerebrospinal fluid pressure does not intensify the headache. Amyl nitrite, however, relieves the headache during the short period of relaxation of the peripheral and splanchnic vascular tone. While the present data, due to the inadequacy of present methods, must necessarily be inconclusive, there are numerous findings that would be in accord with the hypothesis that this headache might result from an increase in effective arterial blood volume without a corresponding diminution in peripheral vascular tone, namely, the gradual onset of cerebral fullness, hyperemia of the ears, lowered serum proteins, the occurrence upon withdrawal of a diuretic drug, and the response to drugs and physical measures affecting headache.

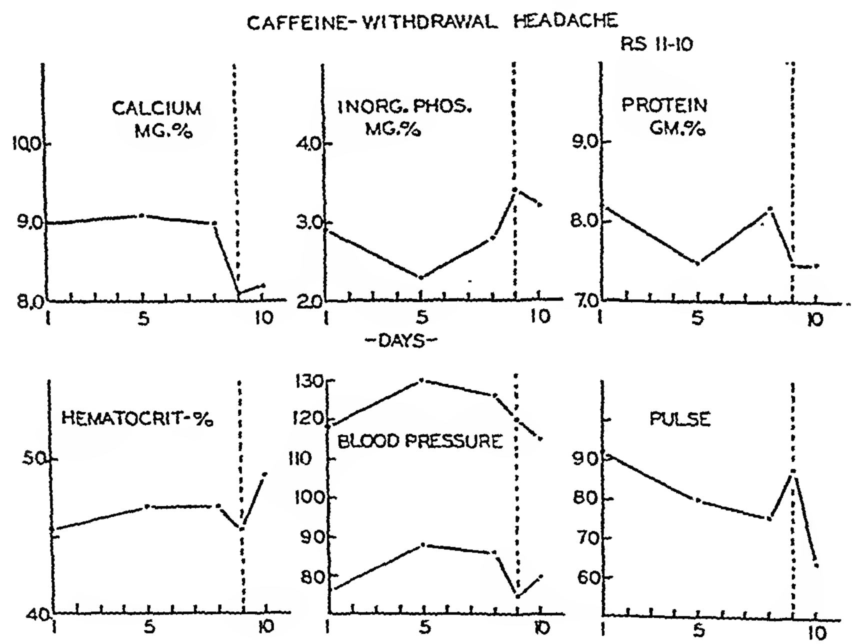


Fig. 3.

It is known from the work of Wolff[14, 15] that many headaches, including histamine and migraine headaches, are accompanied by an increased excursion of the cerebral blood vessels. This may occur reflexly or passively by changes in the volume or pressure of any one of the three semifluid media within the cranial vault, namely, the cerebrospinal fluid, the brain substance, or the blood. Since the first two media are relatively constant, and since the functional pathology of most headaches is known to reside in the vascular wall, the cause of the pain must be either a reflex dilatation of the blood vessels or an increased excursion of the vessels due to the two other factors which could affect the caliber of the cranial blood vessels, namely, the arterial blood volume or the pressure of the surrounding media. Bazett[16] has estimated the *arterial* blood volume from

anatomic measurements of the mesenteric vessels, and his observations indicate that the arteries probably contain a relatively small proportion (perhaps less than 20 per cent) of the total blood in a given region of the circulation. Thus, while the changes in blood volume demonstrated in this study are small, they may have exaggerated significance when combined with changes in cardiac output and peripheral vascular tone.

Since all factors must be considered, there remains the possibility that this headache might be due to direct or reflex dilatation of the cerebral vessels by the withdrawal of caffeine. Hirschfelder[17] has shown that caffeine tends to dilate the cerebral vessels if given intravenously. Oral caffeine is, however, believed to constrict the cerebral vessels, and this has been correlated by Sutherland and Wolff[18] with the observation that caffeine decreases the excursion of the cerebral blood vessels when used in the treatment of migraine headache.

The caffeine-withdrawal headache probably typifies one of the so-called "common headaches." In this respect it may be of value as an experimental headache. It may also provide a means of studying analgesic drugs by affording a method of producing predictable headaches. This study also provides a plausible explanation for the hitherto empirical addition of caffeine to many headache remedies.

The authors wish to express gratitude to the numerous subjects who aided in these experiments.

SUMMARY

In 55 per cent of thirty-eight trials on twenty-two subjects, headache as extreme in severity as the subjects had ever experienced was produced by the sudden withdrawal of caffeine. In 29 per cent of the trials the headache was definite, but did not require treatment. In 16 per cent of the experiments little or no headache resulted. The headache is without scotomas, slow in onset, central in origin, becoming generalized after four to six hours; and it may be accompanied by nausea and vomiting. In migraine subjects the headache differs from their typical migraine headache. The blood studies indicate that a lowered serum calcium, an elevated serum phosphorus, and possibly an increase in blood volume accompany the headache.

REFERENCES

1. Dreisbach, R. H.: Experimental Caffeine Withdrawal Headache, Proc., J. Pharmacol. & Exper. Therap. 69: 11, 1940.
2. Leake, C. D., Loevenhart, A. S., and Muehlberger, C. W.: Dilatation of Cerebral Blood Vessels as Factor in Headache, J. A. M. A. 88: 1076, 1927.
3. Pickering, G. W.: Observations on Mechanism of Headache Produced by Histamine, Clin. Sc. 1: 77, 1933.
4. Clark, D. D., Hough, H., and Wolff, H. G.: Experimental Studies on Headache: Observations on Histamine Headache, Proc. A. Research Nerv. & Ment. Dis. 15: 417, 1934.
5. Schumacher, G. A., Ray, B. S., and Wolff, H. G.: Experimental Studies on Headache. Further Analysis of Histamine Headache and Its Pain Pathways, Arch. Neurol. & Psychiat. 44: 701, 1940.
6. Forbes, H. S., Cobb, S., and Fremont-Smith, F.: Cerebral Edema and Headache Following Carbon Monoxide Asphyxia, Arch. Neurol. & Psychiat. 11: 264, 1924.
7. Pfeiffer, C.: Calcium and Potassium in the Etiology and Treatment of Functional Disorders and Migraine Headaches, Proc., J. Pharmacol. & Exper. Therap. 69: 297, 1940.
8. Inskeep, L. D.: Etiology and Treatment of Migraine, Northwest Med. 32: 67, 1933.

9. Norman, G. F.: Blood Calcium: Its Relation to Certain So-called Angiospastic Conditions, West. J. Surg. 46: 553, 1938.
10. Norman, G. F.: Hypocalcemia; Its Relationship to Migraine, J. A. M. A. 102: 529, 1934.
11. Conwell, D. V., and Kurth, C. T.: Clinical Approach to Migraine Problem, J. Kansas M. Soc. 41: 413, 1940.
12. Pfeiffer, C., Dreisbach, R. H., Glass, H. G., and Roby, C. C.: Hemoconcentration Accompanied by Low Serum Calcium in Vagotomized, Parathyroidectomized Dogs, Endocrinology 27: 818, 1940.
13. Eddy, N. B., and Downs, A. W.: Tolerance and Cross-Tolerance in Human Subject to Diuretic Effect of Caffeine, Theobromine, and Theophylline, J. Pharmacol. & Exper. Therap. 33: 167, 1928.
14. Graham, J. R., and Wolff, H. G.: Mechanism of Migraine Headache and Action of Ergotamine Tartrate, Proc. A. Research Nerv. & Ment. Dis. 18: 638, 1937.
15. Wolff, H. G.: Headache and Cranial Arteries, Tr. A. Am. Physicians 53: 193, 1938.
16. Bazett, H. C.: Bard-Macleod's Physiology in Modern Medicine, ed. 9, St. Louis, 1941, The C. V. Mosby Co., page 341.
17. Hirschfelder, A. D.: Effect of Drugs Upon the Vessels of the Pia Mater and Retina, Proc., J. Pharmacol. & Exper. Therap. 6: 59, 1915.
18. Sutherland, A. M., and Wolff, H. G.: Experimental Studies on Headache. Further Analysis of Mechanism of Headache in Migraine, Hypertension, and Fever, Arch. Neurol. & Psychiat. 44: 929, 1940.

THE ETIOLOGY OF THE MIGRAINE SYNDROME—A PHYSIOLOGIC APPROACH*

CARL PFEIFFER,† PH.D., M.D., ROBERT H. DREISBACH, PH.D., CHARLES C. ROBY, PH.D., AND HOWARD G. GLASS, B.A., CHICAGO, ILL.

THE possibility that blood volume changes might participate in the etiology of migraine headaches has not hitherto been investigated, although Lennox and Leonhardt[1] in 1937 suggested that alterations of "blood concentration" might play a dominant role in migraine headache. Established factors in the migraine syndrome which might be correlated with changes in blood volume are:

1. Onset at puberty and frequent relief at the menopause (correlating with onset and waning of cyclic activity of the sex hormones with their salt- and water-retaining properties).

2. Greater incidence in the female than in the male due to greater salt- and water-retaining power of estrogens compared to androgens.[2]

3. Menstrual migraine (perhaps due to sudden decrease in the estrogens with their salt- and water-retaining activity).

4. Relief of migraine by pregnancy due to the normal, concomitant increase in the blood volume with a high, maintained estrogen level.

5. Partial relief of the headache by the coal tar analgesics[3] and thyroid[4] therapy, both of which are known to produce a relative hydremia.

6. Marked relief by long-acting vasoconstrictor drugs, such as ergotamine tartrate and benzedrine sulfate (amphetamine).

*From the Departments of Pharmacology of Wayne University and the University of Chicago Medical Schools.
†Lieutenant (M.C.), U.S.N.R.
Received for publication, November 16, 1942.

7. The occurrence of the headache with relaxation or a relative "let down" in activity.

These factors might be ascribed to an accompanying relaxation of peripheral-vascular tone without an increase in blood volume. Any altered physiology due to hereditary anomalies of the cerebral arteries would be precipitated at this time because of the decreased blood supply to the cranial area. It was the purpose of this study to determine whether headache of the migraine type is accompanied by relative changes in blood volume; at the same time other data, such as the blood pressure, pulse, serum calcium, phosphorus, and potassium, were obtained.

EXPERIMENTAL

1. *Relaxation Headache.*—The typical Sunday morning headache of the business man, the Monday headache of the clergy, the day-off headache of the nurse, and the post-examination headache of the student may all come under this classification. In order to determine if blood volume or electrolyte changes were involved in relaxation headaches, medical and graduate students were asked to come to the laboratory for blood sampling whenever they had a spontaneous headache. At that time a careful history was taken of the preceding sequence of sleep and activity to correlate any possible relaxation factor. In a series of twenty-six headaches studied, nine could be definitely correlated with relaxation (such as sleeping late). Blood samples were taken and analyzed for serum calcium, potassium, inorganic phosphorus, and proteins. The pulse, the reclining blood pressure (average of 3 to 4 trials), and hematocrits were also determined. A second series of observations and samples was taken after relief of the headache by caffeine and acetylsalicylic acid. If this therapy did not relieve the headache, the second blood sample was not obtained until one or two days had elapsed. Table I summarizes the findings in the nine headaches in which the subjects gave a history of relative relaxation. Ordinarily the serum proteins are the best index of changes in the blood volume, since they are usually constant. A rise in the serum proteins indicates a decrease in the blood volume at the time of the headache. Conversely, a lowered serum protein indicates a hemodilution. The hematocrit is much less reliable because it is changed by mobilization or storage of the red blood cells.

It is of interest to note that half of these subjects with relaxation headache have an appreciable rise in diastolic blood pressure at the time of the headache. With only two exceptions the change in the serum proteins indicates a relative hemoconcentration at the time of the headache. In the case of B.V. the first headache followed sleeping late after a hurried week-end motor trip. Accordingly, the same degree of sleepless activity was again artificially simulated, and the subject again slept late. Except for the blood pressure and the degree of the headache, the findings were almost identical. The first headache was, however, more severe, which happening might in some way be associated with the higher diastolic blood pressure.

2. *Migraine Headache.*—In a similar fashion, blood samples were obtained from migrainous patients suffering from typical migraine headache. Table II summarizes the findings in ten such headaches. These observations differ from

the findings in the relaxation type of headache in that five subjects showed a marked hypotensive level of blood pressure at the time of the headache with a return to normal on relief of the headache. The other six showed higher serum protein levels, thus indicating relative hemoconcentration at the time of the headache. In those cases that had no actual volume change, a relative inadequacy of the effective arterial blood volume is suggested by the decreased blood pressure, indicating a decreased peripheral-vascular tone. There was evidence of a lengthened blood-clotting time during the headache.

TABLE I

RELAXATION HEADACHE

	CA MG. PER 100 C.C.	P MG. PER 100 C.C.	K MG. PER 100 C.C.	PROT. GM. PER 100 C.C.	BLOOD PRESSURE	PULSE	HEMATOCRIT %	
1. B. V. 6/27	9.5	--	--	8.52	130/94	92	50.5	Headache
	9.5	--	--	8.31	134/84	88	50.6	Normal
2. B. V. 7/2	9.4	--	--	8.60	122/76	92	47.5	Headache
	9.2	--	--	8.30	116/78	96	49.2	Normal
3. L. I. 2/7	8.5	3.8	16.0	9.10	140/94	88	47.4	Headache
	--	--	17.5	8.17	138/98	82	48.0	Normal
4. S. S. 12/6	8.8	2.2	--	8.30	122/90	72	47.5	Headache
	9.0	2.3	--	8.60	110/88	68	48.3	Normal
5. R. S. 6/22	9.8	3.6	--	8.10	152/102	88	46.0	Headache
	8.7	2.6	--	7.70	122/85	68	44.2	Normal
6. I. I. 4/20	9.8	--	15.6	8.20	140/98	96	54.6	Headache
	9.8	--	17.9	8.00	126/88	102	45.5	Normal
7. R. D. 11/19	9.9	3.2	--	8.50	108/78	62	50.5	Headache
	9.6	3.7	--	7.75	114/78	60	47.0	Normal
8. P. S. 1/24	9.9	3.5	16.0	9.50	150/100	88	49.0	Headache
	9.6	3.6	16.0	8.80	126/80	54	43.5	Normal
9. R. D. 1/5	9.3	--	--	8.10	122/70	80	48.0	Headache
	9.4	3.5	--	8.10	112/68	56	47.1	Normal
Mean	9.6			8.55	132/89	84	49.0	Headache
	9.4			8.19	122/83	76	47.0	Normal

3. *Menstrual Migraine.*—A few patients with menstrual migraine have two types of headache. The first is a dull, generalized headache which may occur during the week preceding menstruation at the height of the estrogenic salt and water retention and may be due to a relative hemodilution. The second type, a typical migraine syndrome, occurs around the first day of menstruation and is perhaps due to the quick rebound phenomenon described by Harrop.[2] Table III summarizes the findings in five menstrual migraine headaches. It is evident again from these data that the only common factor at the time of the menstrual migraine headache is a decreased blood volume as indicated by a relatively higher serum protein level at the time of the headache. The subject

A. U. is of interest in that the menstrual migraine is accompanied by the same lowering of blood pressure which characterized a previous migraine attack that did not occur at menstruation. The average hematocrit is distorted by the final normal sample in No. 5 which was obtained two weeks after the headache. Menstrual blood loss might also affect the hematocrit readings at the time of the headache.

TABLE II

MIGRAINE HEADACHE

	CA MG. PER 100 C.C.	P MG. PER 100 C.C.	K MG. PER 100 C.C.	PROT. GM. PER 100 C.C.	BLOOD PRESSURE	PULSE	C.T.	HEMATOCRIT %
1. A. U. 4/8	10.0 9.8	-- --	16.8 --	8.20 7.90	95/70 105/75	84 84	4.50 4.20	42.5 Headache 41.0 Normal
2. M. M. 6/13	9.8 9.8	-- --	-- --	9.20 8.31	138/98 124/92	82 76	4.20 3.70	48.5 Headache 48.5 Normal
3. G. C. 4/24	10.8 10.0	-- --	14.4 14.8	8.50 8.20	134/90 118/80	104 88	3.80 3.80	48.5 Headache 45.0 Normal
4. B. O. 5/19	9.3 9.9	-- --	19.1 23.4	8.81 7.40	80/58 115/84	68 58	4.50 4.50	45.0 Headache 43.2 Normal
5. B. O. 6/26	9.4 9.6	-- --	25.3 28.8	8.12 8.10	96/66 126/92	70 70	4.75 4.50	43.0 Headache 43.0 Normal
6. B. O. 8/2	9.4 9.6	3.0 2.5	-- --	8.28 8.36	90/62 108/80	84 64	6.00 4.40	44.0 Headache 41.2 Normal
7. C. H. 10/25	8.4 9.1	4.0 --	-- --	7.94 7.96	90/70 125/85	76 96	-- --	45.5 Headache 48.2 Normal
8. C. A. 8/20	9.8 9.4	-- --	-- --	9.32 7.80	120/85 115/92	80 100	-- 4.70	47.0 Headache* 44.0 Normal
9. L. U. 5/12	8.9 8.6	-- --	16.4 16.4	8.30 8.00	120/75 118/88	106 76	-- --	48.5 Headache 46.7 Normal†
10. C. A. 4/22	10.0 8.9	2.5 2.5	13.6 12.9	8.44 8.17	135/95 140/95	114 108	-- --	47.2 Headache 46.0 Normal
Mean	9.6 9.5			8.51 8.02	110/77 119/86	87 82	4.62 4.18	46.0 Headache 44.7 Normal

*Sample taken after vomiting.
†One hour later.

DISCUSSION

While the extensive studies of Graham and Wolff and their colleagues[5] have indicated that cerebral pain is vascular in origin, they have not explained why the cerebral arteries should suddenly start dilating or why ergotamine, which acts mainly on the peripheral blood vessels, should decrease the excursion of the cerebral blood vessels. Our studies indicate that the excursion of the cerebral blood vessels may be traced to changes in arterial blood volume without compensatory changes in the peripheral vascular tone.

TABLE III

MENSTRUAL MIGRAINE HEADACHE

	CA MG. PER 100 C.C.	P MG. PER 100 C.C.	K MG. PER 100 C.C.	PROT. GM. PER 100 C.C.	BLOOD PRESSURE	PULSE	HEMATOCRIT %	
1. A. U. 2/27/39	9.3	--	19.1	8.00	94/74	84	42.0	Headache
	9.5	--	19.9	7.70	100/74	76	40.5	Normal
2. A. U. 3/22/39	9.9	3.0	--	8.00	98/68	92	42.0	Headache
	9.7	3.7	--	7.90	105/75	84	41.0	Normal
3. E. S. 11/22/38	8.5	2.8	--	7.85	122/82	68	43.2	Headache
	9.1	--	18.7	7.10	115/76	64	43.3	Normal
4. W. 2/8/39	9.0	--	17.5	8.70	118/80	76	42.2	Headache
	9.0	--	--	8.00	120/72	88	40.3	Normal
5. M. S. 12/16/39	9.8	3.8	--	8.94	120/85	80	42.0	Headache
	9.2	4.4	--	8.30	114/85	80	46.0?	Normal
Mean	9.3			8.30			42.3	Headache
	9.3			7.80			42.2	Normal

TABLE IV

COMPARISON OF BLOOD FINDINGS—CAFFEINE-WITHDRAWAL HEADACHE AND MIGRAINE HEADACHE

		BEFORE	DURING	AFTER
Serum Calcium	C. W. H. (17)	9.5 ± .4*	9.1 ± .4	9.4 ± .8
	Migraine (15)		9.5 ± .6	9.4 ± .4
Serum Phosphorus	C. W. H. (17)	3.2 ± .5*	3.6 ± .5	3.4 ± .4
	Migraine (15)		3.1 ± .5	3.2 ± .7
Serum Proteins	C. W. H. (17)	8.2 ± .4†	7.9 ± .4	8.1 ± .5
	Migraine (15)		8.4 ± .4	7.8 ± .3
Hematocrits	C. W. H. (17)	48.2 ± 2	46.7 ± 2	47.9 ± 2
	Migraine (15)		44.7 ± 2	43.8 ± 2

*Mg. per 100 c.c. ± Standard deviation.
†Gm. per cent ± Standard deviation.

The numbers in parentheses represent the number of subjects and patients used in compiling the respective means. The change in serum calcium and phosphorus found in subjects with caffeine-withdrawal headaches does not occur in migraine headaches. The blood volume trend, as judged by the changes in the hematocrits and serum proteins, is opposite in the two headaches.

In 1927 Loevenhart et al.[6] postulated that headache could arise from changes in volume or pressure of any one of the three fluid or semifluid media within the rigid confines of the skull. They defined these three semifluid media as the blood, the brain, and the cerebrospinal fluid. Headache resulting from changes in the volume of the brain and cerebrospinal fluid is well known and self-explanatory. In order to complete and clarify the discussion, we have summarized in Fig. 1 the known hydrodynamic factors which might possibly be involved in all types of headache. The pain-sensitive structures within the cranial vault, namely, the cranial blood vessels (arteries and veins), the dura, the tentorium cerebelli and the falx cerebri, are depicted. The various fluid pressure forces which might cause an increased excursion of the blood vessels or an abnormal stretch of the supporting membranes are also represented. Since the headache due to changes in cerebrospinal fluid volume and actual

brain volume is self-explanatory, the discussion will be limited to the changes in effective blood volume and peripheral vascular tone. While known data are not available on effective blood volume or cardiac output changes in nitrite or histamine headache, it is suspected that since these headaches do not occur

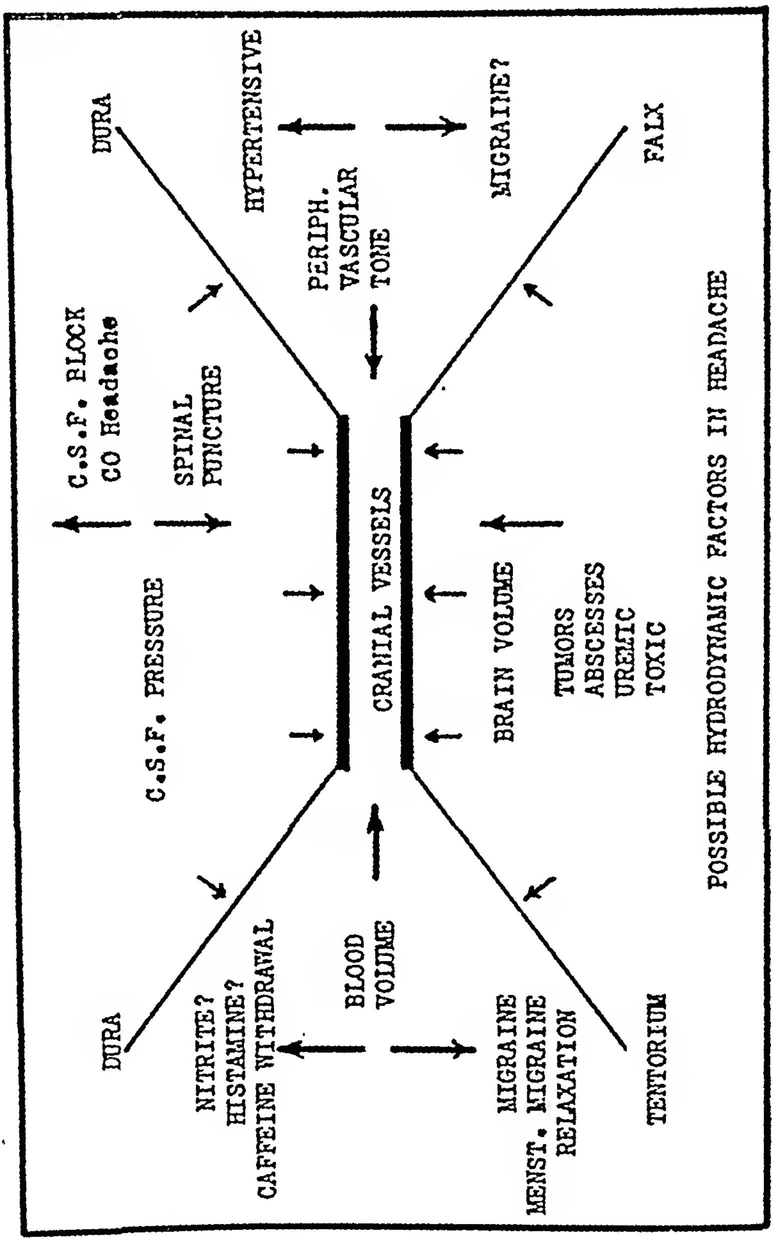


Fig. 1.

at the time of the hypotension from the drugs, they may be connected with the rise in blood pressure following the period of hypotension. If an increase in cardiac output occurs during the hypotensive period, the headache could be caused by a relative increase in the effective arterial blood volume at a time when the peripheral blood vessels are constricting. The data on "caffeine-withdrawal" headache[7] support the theory that this type of headache is accom-

panied by a relative increase in the effective blood volume. Conversely, these studies have substantiated the hypothesis that headache of the migraine and relaxation types is accompanied by a relative diminution in the blood volume.

Hypertensive patients are known to be frequently afflicted with so-called "tension headache." Experimental subjects in this laboratory have experienced similar headache with large doses of ephedrine or neosynephrin. It has been our experience that severe, persistent, frontal headache (occasionally with nausea and vomiting) occurs when normal subjects are given intramuscular doses of neosynephrin HCl in excess of 10 milligrams. This is relieved temporarily by inhalations of amyl nitrite; but complete relief does not occur until the blood pressure returns to normal. This could be accounted for by a sudden increase in peripheral vascular tone without an equal diminution in effective blood volume and cardiac output. A portion of the data in this study supports the theory that some migraine headaches may be accompanied by a marked decrease in peripheral vascular tone, and this is reflected in the semi-shocklike level of blood pressure obtained at the time of the headache.

The treatment of migraine headache should, then, be directed at increasing the blood volume or restoring an intense peripheral vascular tone by strong smooth muscle stimulants, such as ergotamine tartrate or amphetamine sulfate. When this is effectively accomplished, the excessive excursion of the cerebral blood vessels subsides with subjective relief.

SUMMARY

Data are presented which justify separation from the so-called "common headache" of two new types, namely, relaxation headache and "caffeine-withdrawal headache." The former is accompanied by a decreased blood volume, and the latter may be ascribed to a relative increase in the effective arterial blood volume. No consistent blood electrolyte changes occur with relaxation or migraine headache. The determination of the serum proteins and hematocrit during and after migraine headache indicates that the migraine syndrome is accompanied by a relative hemoconcentration. Many other known factors in this syndrome can be correlated with changes in blood volume and peripheral vascular tone.

REFERENCES

1. Lennox, W. G., and Leonhardt, H. C.: Flow and Concentration of Blood as Influenced by Ergot Alkaloids and as Influencing Migraine, Ann. Int. Med. 11: 663, 1937.
2. Harrop, G. A., and Thorn, G. W.: Influence of Sex Hormones on Salt and Water Metabolism. Tr. A. Am. Physicians 52: 164, 1937.
3. Barbour, H. G., and Hermann, J. B.: Relation of Dextrose and Water Content of the Blood to Antipyretic Drug Action, J. Pharmacol. & Exper. Therap. 18: 165, 1921.
4. Friedlander, M., Laskey, N., and Silbert, S.: Effect of Estrogenic Substance on Blood Volume, Endocrinology 20: 329, 1936.
5. Graham, J. R., and Wolff, H. G.: Mechanism of Migraine Headache and Action of Ergotamine Tartrate, Proc. A. Research Nerv. & Mental Dis. 18: 638, 1937.
   Wolff, H. G.: Mechanism of Migraine Headache and Action of Ergotamine Tartrate, Tr. As. Amer. Phys. 53: 193, 1938. Arch. Neurol. & Psychiat. 39: 737, 1938.
6. Leake, C. D., Loevenhart, A. S., and Muehlberger, C. W.: Dilatation of Cerebral Blood Vessels as Factor in Headache, J. A. M. A. 88: 1076, 1927.
7. Dreisbach, R. H.: Experimental Caffeine Withdrawal Headache, Proc., J. Pharmacol. & Exper. Therap. 69: 283, 1940.

CHRONIC BRUCELLOSAL TYPE OF ANKYLOSING SPONDYLITIS

E. GOLDFAIN, M.D., OKLAHOMA CITY, OKLA.

DURING the past four years routine tests have been done on a number of patients with ankylosing spondylitis who came to the office for treatment. These tests, in addition to others, consisted of agglutination and opsonic index and skin test for brucellosis, bacterial complement fixation test with several strains of streptococcus antigens, especially hemolytic, viridans, nonhemolytic, and alpha prime *Streptococcus viridans;* and skin tests with a number of streptococcus strains, such as the hemolytic, viridans, alpha prime viridans, nonhemolytic, and anhemolytic streptococcus types.

In all there have been eighteen cases of *ankylosing spondylitis.* Of the eighteen patients two were women, indicating the usual ratio observed in ankylosing spondylitis of male to female, approximately 8 to 1.

Five patients gave quite positive skin, agglutination, and opsonic index tests for brucellosis. Rather interesting it was that those who gave strongly positive brucellosis tests in general gave essentially negative results on being tested for streptococcal antibodies and by skin test. Each patient had had his symptoms for quite some period of time. A diagnosis of ankylosing spondylitis was based upon a characteristic history, proper physical findings, and the demonstration by x-ray of changes in the vertebral ligaments indicative of ankylosing spondylitis, as well as of sacroiliac joint involvement.

Agglutination tests for brucellosis were carried out by the rapid plate method. The opsonic index test was done according to the method as elaborated by Huddleson and others; the skin test was carried out by injecting 0.1 c.c. of normal salt solution containing 10 million brucella organisms.

Streptococcal antibody tests were done in each case. It was felt that streptococcal skin tests should be performed in such a way that there would be no question, when a positive reaction was obtained, that definite hypersensitivity as a result of either past or present infection against the streptococcal strains was present. One-tenth c.c. of normal saline solution containing 10,000 organisms of the streptococci was injected intradermally, and observation of the skin test reaction was carried out over a period of at least forty-eight hours to as much as seventy-two or ninety-six hours.

A red cell sedimentation test was done in every case.

A review of the literature in respect to complications of either acute or chronic brucellosis, but especially of chronic or convalescent brucellosis, revealed some very pertinent findings in the foreign and American literature.

W. A. Bishop, Jr.,[1] makes some of the following important observations and quotes an extended review of the literature in his article. The result of his survey reveals:

Received for publication, November 16, 1942.

[1]J. Bone & Joint Surg. 21: 665, 1939.

1. "Spinal localization is the most common bone and joint complication of brucellosis."

2. "Localization may appear during any phase of the disease. Localization may be independent of a history of previous ailment. Localization as a complication is more common in the convalescent period."

3. He quotes Serio: "Local recurrence of the brucellosis is probably due to a lack of immunization to the organism."

4. "Localization occurs most often in the lumbar area."

5. "Frequent locomotor symptoms of chronic brucellosis are those of arthralgia, joint effusion, joint swelling, or a combination of arthralgia, joint effusion and joint swelling."

6. "The pathology has usually been described as an epiphysitis. Abscesses of vertebral bodies and/or vertebral discs have been described."

Bishop also quotes:

"Under pathology Feldman and Olson described in the hog irregular abscesses in the vertebral bodies and intervertebral discs. They were able to recover the organisms from ten of these cases. They described 0.5 to 3 cm. grayish-white, thick, pasty or caseous localized areas and occasionally sequestra. Certain lesions presented a crescent-shaped caplike inflammatory proliferation of osteogenic tissue bridging the intervertebral space on the ventral surface of the affected vertebrae. They concluded that the amount of ossification is variable depending on the duration of the lesion."

Bishop—"Their gross description of these lesions in hogs is compatible with the x-ray picture in human cases."

Bishop also quoted Serio as stating that chronic bony spurs on the anterior surface of the vertebral bodies would be noted as a part of the pathology and that occasionally calcification changes in focal areas would appear in the vertebral bodies instead of decalcification as is seen in tuberculosis. In other words, a characteristic zone of sclerosis instead of demineralization may occur at the original site of a brucellosis focus.

Bishop—"Serio states that these sclerotic changes accompany the infiltrative process, which infiltrative process gradually extends and may involve the entire vertebral body, and at times various processes of the vertebrae." Bishop also quotes: "There is variability of site and extent of lesions of the brucellosal complications in the spine. They may be confined to the disc and yet no narrowing of the disc or only very mild narrowing of the disc may occur. Often the vertebral body is normal or it may often be sclerotic or irregular. The original lesion may be masked by concomitant periosteal reaction, such as lipping or spurs on the anterior margin of the vertebrae." Bishop also makes specific mention from the literature of the fact *that occasionally an anteroposterior view of the spine will show the sclerotic processes following the contour of the intervertebral disc.* He also states that in cases in which the condition is more advanced constant and characteristic x-ray changes may be present as follows: "That all showed a reactive change, increased calcification of the bodies, circumscribed or diffuse, with proliferation of bone spurs across the interspace. Occasionally in the more severe cases deformity of vertebral body or *involvement*

of the articular facets will occur. Bony fusion of the bridges between the adjacent bodies is a common finding."

Bishop further quotes: "Hardy and Simpson, independently of each other, reported that one-third of patients with brucellosis had severe arthralgia. Straehm, in a thesis on an epidemic of undulant fever reported occurring in South Africa in 1911, recorded 268 cases in which joint swelling and effusion occurred in 15.3 per cent."

The symptoms of ankylosing spondylitis as a complication to chronic brucellosis are those of a case of infectious spondylitis in which pronounced rigidity of the spine, either locally or throughout its entire extent, plus either local or diffuse muscle spasm, will be present. Rigidity of the spine is especially present if the intervertebral articulations are involved.

The diagnosis of ankylosing spondylitis due to brucellosis must be based upon the history of back pain in patients who have had brucellosis in the past, in those who have some of the symptoms of chronic brucellosis, or in those who give strongly coordinated laboratory and skin test findings indicating the presence of either past or present chronic brucellosis. The agglutination and opsonic index tests are very important, especially the agglutination test. Often, as in other illnesses, the agglutination test may be negative or only very slightly positive, even though the disease process is present in the individual. The skin test should always be strongly positive, though cases are reported where a long-standing case of chronic brucellosis complication may have a negative skin test. The white blood cell count is usually normal or low. The patients may or may not have a temperature. The sedimentation rate may be accelerated. There should be negative findings for typhoid, if symptoms are acute, and negative findings for tuberculosis.

Of course the recovery of the organism from the lesions is the most important confirmatory finding that could be had. But its attempted recovery from chronic cases affords essentially a marked lack of positive results. It is a difficult procedure at the very best to recover organisms from a chronic brucellosis case. The usual findings that are present in a case of ankylosing spondylitis should be present, usually in a male, in the 15-to-45-year age group, with evidence of involvement of the sacroiliac and apophyseal joints, and the presence of calcification of some parts of the spinal ligaments or sacroiliac joints.

The development of the disease syndrome can be divided into three phases, the first phase being that of inexplainable symptoms and known as the prespondylitic phase; the second phase being one in which sacroiliac joint involvement can be demonstrated; and the third phase that in which definite and marked spinal rigidity is noted. The x-ray should be positive during the second and third phases. It will be negative during the first phase.

PATHOLOGY

Gunnar Edstrom[2] quotes the pathology of ankylosing spondylitis as being a chronic synovitis and tendinitis with round cell infiltration, hyperemia, and proliferation of the connecting tissue. These pathologic findings could be noted in any instance of an infection, whether such infection be by one or another known or unknown organism.

[2]Acta med. Scandinav. 101: 396, 1940.

T. Lloyd Tyson[3] describes the pathology as "a primary synovitis of the intervertebral joints with subsequent calcification of the ligamentous structures and eventual rigidity of the spine." The synovitis of the small intervertebral joints may be accompanied by osteoporosis of the vertebral bodies. There is present also at all times synovitis of the sacroiliac joints. Synovitis of the costovertebral joints may be present. Later calcification of the long ligaments, of the ligamentum flavum, and the ligaments at the lateral border of the intervertebral discs will occur. He states that the pelvic and shoulder girdles may be affected. He notes especially that the small joints, when they are involved, are of a typical rheumatoid arthritis involvement. This observation was also reported, in the instance of two cases, by Goldfain.[4]

Jacques Forestier,[5] under the heading "The Importance of Sacro-Iliac Changes in the Early Diagnosis of Ankylosing Spondylitis," states: "Atrophic arthritis of the joints between the facets of the vertebrae in the inflammatory stage is followed by bony ankylosis." Later ossification of adjoining ligaments (ligamenta flava) and anterior and posterior vertebral ligaments occurs. He stresses the importance of noting by x-ray the presence of early changes in the sacroiliac joints. His description of the x-ray changes in the sacroiliac joints would seem to be the description of changes that are often seen in joints having rheumatoid arthritis type of pathology; namely, first there appears marginal decalcification, later there appear pyknotic formations, and finally in the last stage total synostosis.

TABLE I

CHRONIC BRUCELLOSIS SPONDYLITIS, ANKYLOSING TYPE

PATIENT	DIAGNOSIS	BRUCELLA AGG'N	BRUCELLA OPSONIC INDEX	BRUCELLA SKIN TEST	STREP. B.C.F. TEST	STREP. SKIN TEST	SED. TEST
No. 1, C. A. W.	Ankylosing spondylitis (Marie-Strümpell)	Complete 1-200	40% moderate	+4	-	-	-
No. 2, R. E. R.	Ankylosing spondylitis (Marie-Strümpell)	Complete 1-200	12% moderate 52% mild	+4	+4 Hemo. S. +4 APV S. +4 NH S.	1+	+4
No. 3, G. G. (female)	Ankylosing spondylitis (Marie-Strümpell)	Complete 1-200	76% marked	+4	+4 Hemo. S. +4 +4 ?	1+	+2
No. 4, C. B.	Ankylosing spondylitis (Marie-Strümpell)	Marked 1-25 Partial 1-200	4% marked 20% moderate 68% mild	+4	-	-	+1
No. 5, A. R. S.	Ankylosing spondylitis (Marie-Strümpell)	Marked 1-50 Partial 1-200	4% moderate 40% mild	+4	+3 Alpha prime viridans	-	+3

COMMENT ON CASES QUOTED—TABLE I

CASE 1.—C. A. W. This patient lived in a small Oklahoma town where pasteurization was poorly practiced. There was a lack of other findings except a very small infected tonsil

[3] M. Clin. North America 21: 1755, 1937.
[4] J. Lab. & Clin. Med. 27: 168, 1941.
[5] Radiology 33: 389, 1939.

remnant tissue, the removal of which did not help his symptoms. Improvement took place on general medical measures and bacterine therapy.

CASE 2.—R. E. R. The patient's home was in a small town in the panhandle of Texas, where no pasteurization of milk was practiced. In this case 44 tests for three strains of streptococci were present; however, the presence of strongly positive agglutination, opsonic index, and skin tests for brucellosis, plus long duration of trouble and suggestive positive history, justified labeling this case as due to brucellosis.

This patient did not respond well to bacterine therapy.

CASE 3.—G. G. The history of long continued illness, lasting several years, with episodes of fever, residence in a small town in the past, presence of same condition in a brother whom I examined but did not treat, plus strongly positive opsonic index, agglutination, and skin test for brucellosis justifies listing the patient as brucellosal spondylitis, ankylosing type. She responded quite well to bacterine therapy.

CASE 4.—C. B. This patient was living on a farm when his rheumatism began with arthralgias. Removal of his tonsils did not help. Loss of weight and weakness were prominently present. The negative antibody and skin tests for streptococci and strongly positive opsonic index and skin tests and moderately positive agglutination tests justify a diagnosis of chronic brucellosal spondylitis, ankylosing type. The patient's response to treatment has been only moderately favorable thus far.

CASE 5.—A. R. S. This patient lived in the panhandle of Texas. He lived on a farm. Milk was not pasteurized or boiled. His sister also had brucellosis. In a period of eighteen months on routine arthritic management and brucella bacterine his sedimentation test had become normal, and patient is free of symptoms.

CONCLUSIONS

1. Of eighteen cases of ankylosing spondylitis, five can be listed as being caused by chronic brucellosis in this series.

2. Ankylosing spondylitis is a symptom complex. The cause may be an infection which can be either determined, as apparently the five cases quoted could be, or nondeterminable in our present stage of knowledge.

3. Chronic brucellosis, like syphilis or tuberculosis, is protean in its manifestations. It may reveal itself in different ways. Apparently one of the complications of chronic brucellosis may be that of a rheumatoid type of arthritis, which when limited to the spine presents the symptom complex of ankylosing spondylitis.

REFERENCES

Bishop, W. A., Jr.: Vertebral Lesions in Undulant Fever, J. Bone & Joint Surg. 21: 665, 1939.

Calder, Royall M.: Chronic Brucellosis, South. M. J. 32: 451, 1939.

Dawson, Martin Henry: Nelson Loose Leaf Living Medicine, Vol. V, p. 631.

Edstrom, Gunnar: Is Spondylarthritis Ankylopoietica Independent Disease or Rheumatic Syndrome? Acta med. Scandinav. 104: 396, 1940.

Evans, Alice C.: Chronic Brucellosis, J. A. M. A. 103: 665, 1934.

Evans, Alice C.: Studies on Chronic Brucellosis: I. Introduction. Pub. Health Rep. 52: 1072, 1937.

Evans, Alice C.: Studies on Chronic Brucellosis: II. Description of Techniques for Specific Tests, Pub. Health Rep. 52: 1419, 1937.

Evans, Alice C.: The Distribution of Brucella Melitensis Variety Melitensis in the United States, Pub. Health Rep. 52: 295, 1937.

Evans, Alice C., Robinson, Frank H., and Baumgartner, Leona: Studies on Chronic Brucellosis: IV. An Evaluation of the Diagnostic Laboratory Tests, Pub. Health Rep. 53: 1507, 1938.

Forestier, Jacques: The Importance of Sacro-Iliac Changes in the Early Diagnosis of Ankylosing Spondylitis, Radiology 33: 389, 1939.

Foshay, Lee, and LeBlanc, T. J.: The Derivation of an Index Number for the Opsonocytophagic Test, J. Lab. & Clin. Med. 22: 1297, 1937.
Gould, S. E., and Huddleson, I. F.: Diagnostic Methods in Undulant Fever (Brucellosis), With Results of a Survey of 8,124 Persons, J. A. M. A. 109: 1971, 1937.
Hare, Hugh F.: The Diagnosis of Marie-Strümpell Arthritis With Certain Aspects of Treatment, New England J. Med. 223: 702, 1940.
Huddleson, I. F., Munger, Myrtle, Gould, S. E., and Paulson, Doris: A Study of Brucella Infection and Immunity in Humans, Am. J. Trop. Med. 17: 863, 1937.
Keller, Alvin E., Pharris, Crit, and Gaub, W. H.: Undulant Fever: Comparative Value of Certain Diagnostic Tests, South. M. J. 31: 1, 1938.
Lautman, Maurice F.: Chronic Arthritis of the Spine: Its Relation to Rheumatoid Disorders, Arch. Phys. Therapy 18: 630, 1937.
Poston, Mary A.: Studies on Chronic Brucellosis: III. Methods Used in Obtaining Cultures, Pub. Health Rep. 53: 1, 1938.
Simpson, Walter M.: The Diagnosis and Management of Brucellosis, Ann. Int. Med. 15: 408, 1941.
Tyson, T. Lloyd: Spondylitis Ankylopoietica, M. Clin. North America 21: 1755, 1937.
Ziskind, Esther Somerfeld, and Ziskind, Eugene: The Neurologic Aspects of Ankylosing Spondylitis, Bull. Los Angeles Neurol. Soc. 2: 185, 1937.

THE SIGNIFICANCE OF THE GROSS CHARACTER OF THE SPUTUM IN THE PROGNOSIS OF PNEUMOCOCCIC PNEUMONIA*

Arthur W. Frisch, M.D., Alvin E. Price, M.D., and Gordon B. Myers, M.D.
Detroit, Mich.

RECENT studies concerning the important role of edema in the severity and spread of the pneumonic process in the lungs[5, 6, 9, 10, 11, 14] have stimulated our interest in the character of the gross sputum in pneumonia. At necropsy we noted a widespread, heavily infected zone of inflammatory edema in the lungs of patients who had produced large quantities of sputum during life. On the other hand, a narrow, well-defined edema zone at the periphery of the advancing consolidation was present in patients who raised small amounts of more tenacious sputum before death. On the basis of these observations the former cases were classified as "wet" lungs and the latter were designated as "dry" lungs. The characterization of cases as dry or wet lungs was then extended to include all pneumonia patients in whom the gross sputum was examined at daily intervals during the course of the disease.** In the present study of 651 cases, exclusive of Type III,† these findings have been correlated with the number of pneumococci per oil immersion field in Wright-stained smears of sputum,[1, 3] the type of pneumococcus, blood culture, leucocyte count, age of the patient, duration of the disease, the extent of involvement, and the outcome of the pneumonia.

---

*From the Departments of Bacteriology and Internal Medicine, Wayne University College of Medicine and Receiving Hospital, Detroit, Michigan.

Supported by a grant from the Commonwealth Fund to the Michigan Department of Health Laboratories.

Received for publication, November 25, 1942.

**The following report has been restricted to patients who produced rusty sputum. Satisfactory specimens were obtained from 90 per cent of the cases due to Types I through VIII; the remaining 10 per cent, who were either uncooperative or unable to produce rusty sputum, were excluded from the study.

†The outcome in Type III pneumonia is determined primarily by the amount of capsular polysaccharides formed in the sputum and lungs.[3, 4, 11]

METHOD

On admission to the hospital the patients were requested to cough and expectorate into a container. Observations concerning the ease of production and the quantity of sputum were made at the bedside. The specimens were then taken to the laboratory, examined under a strong light, and portions were removed for sputum counts in the manner previously described.[1, 3] The classification of patients as wet or dry lungs was usually, but not always, easy. Those patients who produced homogeneously rusty, watery to thin gelatinous sputum in considerable quantities and with relative ease on coughing were classified as wet lungs. Those who, upon vigorous coughing, raised small amounts of viscid sputum, which was either homogeneously rusty or mixed with mucoid or mucopurulent material, were called dry lungs. In dehydrated patients the final classification was deferred until adequate fluids had been administered. In borderline cases the consistency of the sputum was the chief factor in determining classification.

RESULTS

Studies of 678 cases of pneumococcic pneumonia reported in detail elsewhere[3] have shown that the fatality rate was only 2 per cent when the average number of pneumococci per oil immersion field in Wright-stained smears of sputum did not exceed 10 (Group A). The fatality was 9 per cent when the sputum count ranged between 11 and 30 per field (Group B), 30 per cent when 31 to 75 organisms were present (Group C), and 77 per cent when the number exceeded 75 per field (Group D). The relation of the gross character of the sputum to the microscopic counts and to the outcome of the disease is presented in Table I. The fatality rate was 6 per cent for all cases classified as dry lungs and 23 per cent for those designated as wet lungs. When the comparison was restricted to patients whose sputum counts fell within a similar range, the fatality rates for the dry and wet lungs were essentially the same and were primarily influenced by the number of pneumococci present; however, only 7 per cent of the dry and 41 per cent of the wet lungs had more than 30 pneumococci per field in their sputa. The close relationship between the sputum count and the character of the pneumonic exudate is further emphasized by a consideration of the percentage of total cases within a given sputum group which were classed as dry or wet lungs: thus 85 per cent of the cases in Group A were dry and 15 per cent were designated as wet lungs. Corresponding figures for Group B were 45 and 55 per cent; for Group C, 28 and 72 per cent; for Group D 2 and 98 per cent, respectively. The significance of these data will be discussed.

In a previous study[3] it was also found that type, qualitative bacteremia, leucocyte count, and duration of the disease before therapy were relatively unimportant factors in prognosis when compared with the number of pneumococci in the sputum. Since 80 per cent of the patients with more than 30 organisms per field were characterized as wet lungs, it seemed important to determine the relation between the gross sputum and other prognostic factors. In Table II the cases have been classified according to the presence or absence of bacteremia. Of the total group of 651 patients studied, 59 per cent were dry and 41 per cent were wet lungs. Among those with negative blood cultures, however, the in-

cidence of dry and wet lungs was 75 per cent and 25 per cent, respectively. These figures were reversed in the group with positive blood cultures, where dry lungs were found in only 26 per cent and wet lungs in 74 per cent. It would appear, then, that blood stream invasion may be dependent upon the formation of large amounts of exudate in the edema zone of the lungs.

TABLE I

RELATION OF DRY AND WET LUNGS TO SPUTUM COUNTS

SPUTUM GROUP	DRY LUNG				WET LUNG				TOTAL CASES		
	CASES		DEATHS		CASES		DEATHS				
	NO.	% OF TOTAL	NO.	% FAT.	NO.	% OF TOTAL	NO.	% FAT.	NO.	% DRY	% WET
A 10 or less	262	69	3	1	47	18	2	4	309	85	15
B 11-30	92	24	10	11	111	41	8	7	203	45	55
C 31-75	27	7	7	30	68	25	21	31	95	28	72
D Over 75	1	0	1	100	43	16	32	74	44	2	98
Totals	382	100	21	6	269	100	63	23	651	59	41

TABLE II

RELATION OF DRY AND WET LUNGS TO BACTEREMIA

SPUTUM GROUP	NEGATIVE BLOOD CULTURE		POSITIVE BLOOD CULTURE		TOTAL CASES		
	CASES		CASES			BLOOD CULTURE	
	NO.	% OF TOTAL	NO.	% OF TOTAL	NO.	% NEGATIVE	% POSITIVE
Dry lung	327	75	55	26	382	86	14
Wet lung	112	25	157	74	269	42	58
Totals	439	100	212	100	651	67	33

TABLE III

RELATION OF DRY AND WET LUNGS TO TYPE*

TYPE	DRY CASES		WET CASES	
	NO.	% OF TOTAL	NO.	% OF TOTAL
I	80	54	69	46
II	60	55	48	45
VII	67	60	45	40
VIII	45	57	34	43
Others	130	64	73	36
Totals	382	59	269	41

*Type III not included.

The relationship between the gross character of the sputum and the type of pneumococcus responsible for the disease is shown in Table III. A breakdown of the cases revealed that the percentage of dry and wet lungs in Types I, II, VII, and VIII pneumonia did not deviate significantly from the mean of 59 and 41 per cent, respectively. Thus there was no evidence from this study that the formation of large amounts of exudate in the lung is a property peculiar to certain types of pneumococci.

The relationship between leucocyte counts and the gross character of the sputum is presented in Table IV. Among the total group of 483 cases with leucocytosis the incidence of dry and wet lungs was 64 and 36 per cent, re-

spectively, as compared with 43 and 57 per cent in the 254 patients with leucocyte counts below 10,000 per cubic millimeter. These figures reveal that there is a definite trend toward leucopenia in wet lung cases, but the differences were too small to suggest that a deficit of leucocytes is the chief factor in the formation of the edema zone in the lung.

The relationship between the gross character of the sputum and the duration of the disease on admission to the hospital is shown in Table V. Only 33 per cent of the total cases were seen more than 96 hours after the pneumonia had developed. The incidence of dry and wet lungs, however, remained approximately the same irrespective of the duration of the disease. It was concluded from these data that the length of time the patient was ill played little, if any, role in the formation of large quantities of exudate in the lung.

TABLE IV

RELATION OF DRY AND WET LUNGS TO LEUCOCYTE COUNT

SPUTUM GROUP	10,000 AND OVER		5,000 TO 9,999		LESS THAN 5,000		TOTAL CASES		
	CASES		CASES		CASES		NO.	LEUCOCYTE COUNT	
	NO.	% OF TOTAL	NO.	% OF TOTAL	NO.	% OF TOTAL		% OVER 10,000	% BELOW 10,000
Dry lung	311	64	49	42	16	44	376	83	17
Wet lung	172	36	69	58	20	56	261	66	34
Totals	483	100	118	100	36	6	637	76	18

TABLE V

RELATION OF DRY AND WET LUNGS TO DURATION OF DISEASE BEFORE THERAPY

SPUTUM GROUP	LESS THAN 48 HOURS		48 TO 95 HOURS		96 TO 143 HOURS		144 HOURS AND OVER		TOTAL CASES		
	CASES		CASES		CASES		CASES		NO.	HOURS	
	NO.	% OF TOTAL	NO.	% OF TOTAL	NO.	% OF TOTAL	NO.	% OF TOTAL		% UNDER 96	% OVER 96
Dry lung	124	59	130	59	75	60	47	55	376	68	32
Wet lung	85	41	89	41	50	40	39	45	263	66	34
Totals	209	100	219	100	125	100	86	100	639	67	33

In a previous study[3] it was noted that the degree of involvement and the age of the patient were factors which significantly influenced the fatality rate independent of the sputum count. In the present study, a close correlation was observed between the character of the gross sputum and the extent of consolidation. The incidence of wet lungs was only 34 per cent in the cases with consolidation confined to one lobe as compared to 64 per cent in those with multiple lobe involvement. While the reversal of the percentages was significant (Table VI), it was felt that the multiple lobe involvement, like bacteremia, was the result of, rather than the cause of, the outpouring of exudate into the bronchi and trachea. In this respect the character and quantity of the exudate might have contributed to the higher fatality rate associated with multiple lobe involvement.[3]

An analysis of the gross character of the sputum with reference to age is presented in Table VII. The incidence of wet lungs increased from 32 per cent in patients below 30 years to 44 per cent in those between 30 and 54 and finally to 51 per cent in the older age group. The trend revealed by these figures suggests that the character and quantity of the pneumonic exudate depend to some extent upon the age of the patient.

TABLE VI

RELATION OF DRY AND WET LUNGS TO INVOLVEMENT

SPUTUM GROUP	ONE OR LESS LOBES, CASES: NO.	ONE OR LESS LOBES, CASES: % OF TOTAL	MORE THAN ONE LOBE, CASES: NO.	MORE THAN ONE LOBE, CASES: % OF TOTAL	TOTAL CASES: NO.	TOTAL CASES, LOBES: % ONE OR LESS	TOTAL CASES, LOBES: % MORE THAN ONE
Dry lung	325	66	57	36	382	85	15
Wet lung	169	34	100	64	269	63	37
Totals	494	100	157	100	651	76	24

TABLE VII

RELATION OF DRY AND WET LUNGS TO AGE OF PATIENT

SPUTUM GROUP	AGE 5 TO 29 YEARS, CASES: NO.	AGE 5 TO 29 YEARS, CASES: % OF TOTAL	AGE 30 TO 54 YEARS, CASES: NO.	AGE 30 TO 54 YEARS, CASES: % OF TOTAL	AGE 55 YEARS AND OVER, CASES: NO.	AGE 55 YEARS AND OVER, CASES: % OF TOTAL	TOTAL CASES: NO.	TOTAL CASES, AGE: % UNDER 30	TOTAL CASES, AGE: % OVER 30
Dry lung	124	68	218	56	40	49	382	32	68
Wet lung	58	32	169	44	42	51	269	21	79
Totals	182	100	387	100	82	100	651	28	72

DISCUSSION

The classification of cases according to the gross character of the pneumonic sputum is a simple procedure which appears to be of considerable aid in prognosis. An overwhelming majority of the patients with dry lungs showed less than 30 pneumococci per field (93 per cent), a negative blood culture (86 per cent), a favorable leucocyte response (83 per cent), and consolidation which was confined to a single lobe (85 per cent). The relatively mild nature of the pneumonia which could be predicted from the appearance of the gross sputum was further substantiated by the fact that 94 per cent of the patients recovered irrespective of therapy. On the other hand, 41 per cent of the cases which were designated as wet lungs showed more than 30 pneumococci per field in the sputum; 58 per cent had bacteremia; 34 per cent had leucocyte counts below 10,000; and 37 per cent had involvement of more than one lobe on admission to the hospital. The pneumonia in these patients was more severe as shown by the higher fatality rate (23 per cent). As a general rule the clinical response to sulfonamides was slower in the cases classed as wet lungs.

The observations of Loeschcke[9] first drew attention to the significance of the edema zone in the transfer of pneumococci from one portion of the lung to another. His findings were soon corroborated by other workers.[6, 10, 11, 14] The experiments of Robertson and Hamburger[11] were of special interest to us because

of their conclusion that the severity of experimental pneumonia in the dog was directly related to the wetness of the lung. They believed that "metastatic lesions occur only when the primary lesion is sufficiently edematous to permit escape of fluid exudate through its main bronchus into that of another lobe or lobes, the direction of flow being determined largely by gravity." The bronchi of animals severely ill with extensive pneumonia yielded considerable quantities of a fluid exudate containing from 5 to 50 billion organisms per cubic centimeter. On the other hand, the exudates from animals with well-localized lesions were usually viscid and contained relatively few pneumococci. From these observations, which were substantiated by our own necropsy findings, it seems likely that the amount and gross character of the sputum in human pneumonia reflects the slate of the lung, copious wet sputum indicating an extensive area of inflammatory edema, a dry sputum indicating a more localized lesion. Such a hypothesis furnishes us with a plausible explanation for the high incidence of bacteremia and multiple lobe involvement in patients who have wet lungs, since the presence of a large edema zone would permit adequate opportunity for blood stream invasion or for metastatic spread to other lobes. It seems to us that the wet lung is a more likely cause of bacteremia than the result of the blood stream invasion, as has been suggested by Kempf and Nungester.[7]

The question as to why one patient should produce large quantities of rusty sputum and another relatively little remains unanswered. While an increase in the proportion of wet lungs occurred with advancing age and with leucopenia, it was felt that these were accessory rather than primary factors determining the character of the pneumonic exudate. The close correlation between the number of pneumococci and the nature of the gross sputum may be interpreted in one of two ways. It is possible that the outpouring of fluid into the alveoli represents an allergic response to the presence of a few pneumococci,[7] thus creating an environment which facilitates the rapid multiplication of the organisms; however, it seems more likely that the amount of exudate is directly proportional to the quantity of edema-producing substance elaborated by the large numbers of pneumococci present in the lung. This concept is supported by (a) the work of Sutliff and Friedemann,[12] who demonstrated such an edema-producing substance in filtrates of young pneumococcus cultures, and by (b) the studies of Hamburger and Robertson,[6] in which either localized or diffuse edematous lesions were experimentally induced at will by varying the dosage of pneumococci. The solution to this problem must await further study.

SUMMARY

The gross character of rusty sputum in 651 cases of pneumococci pneumonia was correlated with standard prognostic criteria and the outcome of the disease. As a result of observations made at necropsy, those patients who produced small amounts of a viscid rusty sputum were classified as "dry" lungs, whereas those who raised large quantities of watery to gelatinous, homogeneously rusty sputum were classified as "wet" lungs. The pneumonia in the former group was relatively mild, while the disease in the latter group was considerably more severe, as shown by fatality rates of 6 per cent and 23 per cent, respectively, as well as by significant differences in the number of pneumococci

in the sputum, the extent of consolidation, and the incidence of bacteremia and leucopenia. It was concluded that the gross character of the sputum was of distinct value in the prognosis of pneumococcic pneumonia. The significance of the observations with reference to the pathology of the pulmonary lesions is discussed.

REFERENCES

1. Frisch, A. W.: Sputum Studies in Pneumonia as an Aid in Prognosis, Am. J. Clin. Path. 10: 472, 1940.
2. Frisch, A. W.: Sputum Studies in Type III Pneumonia, Am. J. Clin. Path. 10: 873, 1940.
3. Frisch, A. W., Price, A. E., and Myers, G. B.: The Prognostic Significance of the Number of Pneumococci in the Sputum in Relation to Therapy, Leukocyte Count, Bacteremia, Type, Duration of the Disease, Age, and Degree of Involvement, J. Clin. Investigation 22: 207, 1943.
4. Frisch, A. W., Tripp, J. T., Barrett, C. D., and Pidgeon, B. E.: The Specific Polysaccharide Content of Pneumonic Lungs, J. Exper. Med. 76: 505, 1942.
5. Gunn, F. D., and Nungester, W. J.: Pathogenesis and Histopathology of Experimental Pneumonia in Rats, Arch. Path. 21: 813, 1936.
6. Hamburger, M., and Robertson, O. H.: Studies on the Pathogenesis of Experimental Pneumococcus Pneumonia in the Dog: Secondary Pulmonary Lesions. Relation of Bronchial Obstruction and Distribution of Pneumococci in Their Inception, J. Exper. Med. 72: 261, 1940.
7. Heffron, R.: Pneumonia With Special Reference to Pneumococcus Lobar Pneumonia, New York, 1939, The Commonwealth Fund.
8. Kempf, A. H., and Nungester, W. J.: Production of Pneumonia in Rats by Intravenous Injection of Pneumococci, Proc. Soc. Exper. Biol. & Med. 43: 627, 1940.
9. Loeschcke, H.: Untersuchungen über die kruppöse Pneumonie, Beitr. z. path. Anat. u. z. allg. Path. 86: 201, 1931.
10. Robertson, O. H., Coggeshall, L. T., and Terrell, E. E.: Experimental Pneumococcus Lobar Pneumonia in the Dog: III. Pathogenesis, J. Clin. Investigation 12: 467, 1933.
11. Robertson, O. H., and Hamburger, M.: Studies on the Pathogenesis of Experimental Pneumococcus Pneumonia in the Dog: II. Secondary Pulmonary Lesions. Their Production by Intratracheal and Intrabronchial Injection of Fluid Pneumonic Exudate, J. Exper. Med. 72: 275, 1940.
12. Sutliff, W. D., and Friedemann, T. E.: A Soluble Edema-Producing Substance from the Pneumococcus, J. Immunol. 34: 455, 1938.
13. Tripp, J. T., Frisch, A. W., Barrett, C. D., and Pidgeon, B. E.: The Specific Polysaccharide Content of Pneumonia Sputa, J. Exper. Med. 76: 497, 1942.
14. Wood, W. B., Jr.: Studies on the Mechanism of Recovery in Pneumococcal Pneumonia: I. The Action of Type Specific Antibody Upon the Pulmonary Lesion of Experimental Pneumonia, J. Exper. Med. 73: 201, 1941.

CLINICAL CHEMISTRY

THE COMPARATIVE ACCURACY OF THE CLOSED CIRCUIT BEDSIDE METHOD AND THE OPEN CIRCUIT CHAMBER PROCEDURE FOR THE DETERMINATION OF BASAL METABOLISM*

ROBERT C. LEWIS, PH.D., ALBERTA ILIFF, PH.D., AND ANNA MARIE DUVAL, PH.D.
DENVER, COLO.

SUFFICIENT data have been accumulated by the investigations of the Child Research Council at Denver to establish standards of basal metabolism on children between the ages of 2 and 16 years. The first report of this work by Lewis, Kinsman and Iliff[1] gives the results obtained prior to April, 1936, and presents tentative standards for both boys and girls from 2 to 12 years old inclusive. Since the appearance of this publication, the question has been raised as to whether these standards may be used when determinations are made with a bedside type of apparatus. No report of a comparison of the results obtained with a chamber procedure and with a bedside apparatus has been found in the literature, although several studies[2-4] have been made on adults both with the open circuit and the closed circuit methods but where a mask or a mouthpiece and a nose clip have been used in both cases. In the present study determinations of the basal metabolism of both children and adults made with an open circuit chamber procedure have been compared with those obtained with a closed circuit bedside type of apparatus.

The open circuit chamber method was that used to establish standards of basal metabolism on children of the Child Research Council. The procedure is fully described in the publication of Lewis, Kinsman, and Iliff.[1] It should be noted that with this method a 15 per cent protein metabolism has been assumed and that the calorific value per standard liter of oxygen, which varies with the respiratory quotient found, has been obtained by reference to the chart of Michaelis.[5]

The closed circuit apparatus employed was a No. M 84 McKesson Metabolor, to which the subject was connected by means of a mask (face inhaler) or with a mouthpiece and nose clip. A McKesson face inhaler, a McKesson mouthpiece, and a Sanborn nose clip were the types of accessories used. The mouthpiece and nose clip were used invariably on adults and in about half of the tests on children. Those cases in which the mask (face inhaler) was used on children are indicated by an asterisk (*) in Table I. The quantity of oxygen consumed in

*From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver.

A preliminary report of this study was made at the meetings of the Colorado-Wyoming Academy of Science at Golden, Colorado, in November, 1941 (Iliff, Alberta, Anna Marie Duval and Robert C. Lewis, J. Colo.-Wyo. Acad. Sci. 3: 56, 1942).

Received for publication, December 22, 1942.

a given length of time was measured, and a value of 4.825 calories per standard liter of oxygen was used to calculate the heat production. In order to determine the consumption of oxygen, check tests of four or more minutes in length were procured in all cases. If the oxygen consumption showed a deviation of more than 4 per cent from the mean when two determinations were made, at least one more test was obtained. For three or four tests to be acceptable, it was required that the oxygen consumption in each case fall within 5 or 7 per cent, respectively, of the mean of all tests.

Alcohol check determinations were made on both types of apparatus during this study. With the chamber method these determinations are made routinely about four times a year. The procedure and the results obtained are given in other publications.[1, 6] The amounts of oxygen consumed and of carbon dioxide produced by the burning of a known quantity of alcohol in the chamber have been found to fall within ±2 per cent of the theoretical values. Twenty-nine determinations with the closed circuit apparatus were made during this study by means of the alcohol check apparatus of Jones,[7] and in these the amount of oxygen consumed varied from the theoretical values by −3.1 to +3.0 per cent with an average deviation of +0.4 per cent.

EXPERIMENTAL PROCEDURE

The experimental procedure followed was to make a determination of basal metabolism in the chamber either immediately preceding or immediately following one with the closed circuit bedside apparatus. Such comparisons were made in 65 instances on 36 children (19 boys and 17 girls) between the ages of 2 years, 8 months, and 15 years, 3 months, and in 55 instances on 25 adults (8 men and 17 women). All subjects were in a postabsorptive condition when the tests were performed. The children had had a normal amount of sleep and were brought from home in an automobile by one of the workers in the metabolism laboratory. The adults had had approximately eight hours' sleep on each of the two nights preceding a test and came to the laboratory by automobile.

In the majority of experiments, a determination was made first with the chamber and then with the McKesson Metabolor. After the subject was comfortable and relaxed, the lid of the chamber was closed and, when the preliminary period (thirty minutes for children and forty-five minutes for adults) had elapsed, samples of air were collected for analysis. The lid of the chamber was then raised, and while the subject remained quiet the body temperature, the pulse rate, and the respiratory rate were taken. The determination with the McKesson Metabolor was then made.

When the bedside method was used first, it was necessary for the subject to remain quiet fifteen or twenty minutes longer than when the reverse order was followed. Since many of the children were inclined to become restless when the experimental period was thus prolonged, only four experiments in which the determinations with the closed circuit apparatus preceded that with the chamber were conducted on children. With adults, 23 experiments were made in this sequence. After the subject rested in the chamber for the preliminary period (thirty minutes for children and forty-five minutes for adults), the consumption of oxygen was measured with the McKesson Metabolor. When satisfactory determinations

TABLE I

COMPARATIVE RESULTS OBTAINED FOR THE BASAL METABOLISM OF CHILDREN WITH THE OPEN CIRCUIT CHAMBER PROCEDURE AND THE CLOSED CIRCUIT BEDSIDE APPARATUS (MCKESSON)

SUBJECT, SEX	AGE		WEIGHT (KG.)	HEIGHT (CM.)	BODY SURFACE (SQ. M.)	CAL./HR./SQ. M.		DIFFERENCE (PER CENT)
	YR.	MO.				BY CHAMBER METHOD	BY MCKESSON METHOD	
7(♂)	2	8	13.7	92.8	0.580	47.6	50.7	+6.5
82(♂)‡	4	2	15.2	101.6	0.660	49.6	51.6	+4.0
53(♀)	4	7	17.7	110.6	0.735	48.2	48.2	±0.0
87(♀)	4	8	14.5	102.5	0.615	45.8	44.9	-2.0
	4	11	14.6	101.7	0.650	46.9	48.5	+3.4
67(♀)	4	11	18.8	106.5	0.735	53.9	51.3	-4.8
71(♀)	5	0	15.3	109.8	0.690	49.6	49.4	-0.4
	5	3	16.1	112.3	0.720	49.0	48.5	-1.0
97(♀)	5	4	19.3	113.4	0.780	46.8	49.5	+5.8
37(♀)	5	7	22.2	119.7	0.860	49.3	51.7*	+4.9
	6	2	23.0	124.2	0.895	46.9	46.9	±0.0
46(♀)	5	9	20.5	118.5	0.825	45.1	47.7	+5.8
29(♂)	5	9	21.0	114.7	0.815	55.8	53.5	-4.1
	6	0	21.2	115.8	0.825	53.4	51.9	-2.8
	6	3	21.3	117.6	0.830	53.2	53.5	+0.6
21(♀)	6	1	18.8	113.8	0.775	45.8	46.2*	+0.9
	6	5	18.7	114.2	0.775	45.8	47.7*	+4.1
27(♂)‡	6	7	22.6	121.8	0.875	50.8	49.8*	-2.0
3(♀)	6	7	16.0	112.7	0.715	48.3	50.2	+3.9
	6	11	16.9	114.3	0.740	48.9	49.5	+1.2
23(♂)	6	8	22.9	119.7	0.870	48.1	44.5	-7.5
95(♀)‡	7	7	25.9	124.6	0.940	46.5	44.3	-4.7
	7	10	27.1	126.6	0.970	46.6	46.9	+0.6
93(♂)	7	7	25.1	127.0	0.940	48.4	47.2*	-2.5
	8	1	27.0	130.3	0.985	45.9	44.1*	-3.9
91(♂)	8	0	22.8	127.7	0.910	49.1	48.9*	-0.4
	8	6	23.0	130.6	0.935	45.4	46.1	+1.5
92(♂)	8	5	29.7	137.4	1.070	43.4	44.6	+2.8
6(♂)‡	8	6	25.2	131.8	0.970	47.2	45.9*	-2.8
	8	9	25.6	133.4	0.985	47.0	48.0*	+2.1
J(♂)	9	0	23.4	129.6	0.925	47.7	47.4	-0.6
	9	4	24.7	130.7	0.960	45.2	47.4	+4.9
	9	10	25.4	133.5	0.980	45.5	46.3	+1.8
81(♂)	9	1	26.0	131.5	0.980	46.5	44.7*	-3.9
	9	8	27.6	134.9	1.020	49.4	46.6*	-5.7
83(♂)‡	9	8	30.6	144.4	1.130	41.7	39.7*	-4.8
80(♂)	9	9	22.5	131.2	0.920	43.3	44.3	+2.3
73(♀)	9	11	25.6	130.4	0.970	43.1	44.2*	+2.6

*In these cases a mask was used; in all other cases a mouthpiece and a nose clip were employed.

†In these experiments the determination with the McKesson Metabolor preceded the observation with the chamber procedure.

‡These children required a preliminary period of training before satisfactory determinations were obtained.

TABLE I—CONT'D

SUBJECT, SEX	AGE YR.	AGE MO.	WEIGHT (KG.)	HEIGHT (CM.)	BODY SURFACE (SQ. M.)	CAL./HR./SQ. M. BY CHAMBER METHOD	CAL./HR./SQ. M. BY MC KESSON METHOD	DIFFERENCE (PER CENT)
70(♀)	10	2	34.6	150.0	1.225	38.5	36.7*	-4.7†
	10	3	34.6	149.1	1.225	38.1	37.1*	-2.6†
B(♂)‡	11	2	45.8	149.3	1.405	40.3	42.3*	+5.0
E(♀)	11	5	40.1	147.1	1.260	44.2	41.9*	-5.2
	11	11	46.7	143.3	1.370	39.5	39.5	±0.0
C(♂)	11	8	45.7	154.2	1.375	45.9	48.4*	+5.4
	11	11	46.3	150.7	1.390	48.3	46.6*	-3.5
48(♂)	12	9	40.5	157.9	1.355	42.6	41.7*	-2.1
	13	1	42.2	159.7	1.390	38.4	40.3*	+4.9
12(♂)	12	11	35.6	145.5	1.210	37.1	38.2*	+3.0
	14	4	40.0	153.1	1.315	39.2	41.6*	+6.1
	14	6	40.8	155.1	1.340	43.0	40.0*	-7.0
	14	9	42.4	157.5	1.380	42.7	40.5*	-5.2
34(♀)	13	0	67.7	164.2	1.740	37.5	37.9*	+1.1
20(♂)‡	13	3	42.8	162.0	1.410	42.5	41.4*	-2.6
	13	6	44.7	164.4	1.450	40.9	41.7*	+2.0
33(♀)	13	8	59.4	158.5	1.605	35.8	35.4*	-1.1
	14	0	62.0	160.3	1.645	34.0	35.7*	+5.0
15(♂)‡	14	0	43.1	162.8	1.425	43.0	41.4*	-3.7
	14	3	47.1	165.1	1.495	43.1	42.7	-0.9
	14	5	47.3	166.8	1.510	40.2	40.0	-0.5†
	14	5	47.5	166.7	1.510	38.7	41.3	+6.7
	14	5	47.3	166.8	1.510	39.2	39.4	+0.5†
	14	6	47.5	166.9	1.510	38.9	40.7	+4.6
11(♀)	14	4	45.6	170.7	1.510	37.9	37.8*	-0.3
6(♀)	15	0	45.8	168.6	1.500	33.6	32.9*	-2.1
	15	3	46.3	168.8	1.510	33.4	34.3*	+2.7

had been obtained, the bedside apparatus was disconnected and the lid of the chamber was closed. After allowing a period of fifteen or twenty minutes to permit the carbon dioxide in the chamber to reach equilibrium, samples of air were collected for analysis.

EXPERIMENTAL RESULTS

The experimental results show an excellent agreement between the values obtained by the bedside method and by the chamber procedure. The calories per hour per square meter of body surface found by each of the two methods are given in Table I for children and in Table II for adults. These tables also give the difference between the values obtained by the two methods in each case in terms of percentage deviation from the determination made in the chamber. These deviations are shown graphically in the accompanying histograms (Fig. 1 for children, and Fig. 2 for adults). The results with the closed circuit bedside method show a deviation from those with the open circuit chamber procedure of from −7.5 to +6.7 per cent (mean variation = −0.2 per cent) for children and of from −8.6 to +9.3 per cent (mean variation = +0.1 per cent) for adults. The order in which the determinations were made had no effect on the results, for

TABLE II

COMPARATIVE RESULTS OBTAINED FOR THE BASAL METABOLISM OF ADULTS WITH THE OPEN CIRCUIT CHAMBER PROCEDURE AND THE CLOSED CIRCUIT BEDSIDE APPARATUS (McKESSON)

SUBJECT, SEX	AGE (YEARS)	WEIGHT (KG.)	HEIGHT (CM.)	BODY SURFACE (SQ. M.)	CAL./HR./SQ. M.		DIFFERENCE (PER CENT)
					BY CHAMBER METHOD	BY Mc KESSON METHOD	
HB(♀)	21	59.6	169.5	1.685	30.4	31.0	+2.0
		59.6		1.685	30.0	29.6	-1.3
		59.8		1.685	30.4	29.9	-1.6
BM(♀)	21	53.1	157.4	1.520	31.0	33.5	+8.1
		53.1		1.520	34.4	33.2	-3.5†
		53.7		1.530	35.3	33.0	-6.5
NM(♂)	22	65.0	172.6	1.775	33.7	33.1	-1.8
		65.7		1.780	34.3	32.6	-5.0†
		65.5		1.780	33.3	34.9	+4.8
GM(♂)	22	66.2	183.6	1.870	34.7	36.7	+5.8
		67.5		1.880	35.9	37.5	+4.4†
		67.7		1.885	35.9	36.5	+1.7
FM(♀)	22	50.2	167.2	1.555	30.6	30.6	±0.0†
		50.1		1.550	32.4	32.3	-0.3
LP(♀)	23	61.8	175.6	1.755	30.0	28.8	-4.0
		61.5		1.750	28.6	28.7	+0.3†
AJ(♀)	23	57.8	160.5	1.600	31.7	31.5	-0.6†
		56.9		1.590	30.3	28.9	-4.6
RC(♂)	25	73.0	190.3	1.995	32.7	32.1	-1.8†
		73.4		2.000	31.9	32.1	+0.6
GD(♂)	26	76.6	179.2	1.955	36.1	36.7	+1.7
		76.4		1.950	34.6	33.8	-3.5†
AW(♂)	26	67.2	183.5	1.880	35.3	33.3	-5.7
RW(♂)	26	72.3	178.8	1.900	32.9	31.0	-5.8
DH(♀)	26	57.9	170.9	1.670	30.0	30.5	+1.7
		57.6		1.670	29.5	30.3	+2.7†
AK(♀)	27	60.3	169.4	1.690	29.3	30.8	+5.1
		60.4		1.695	29.6	30.0	+1.4†
AMD(♀)	27	53.8	158.6	1.540	30.1	32.9	+9.3
		53.1		1.530	31.1	33.4	+7.4†
		54.0		1.540	30.5	31.5	+3.3
LW(♀)	27	50.5	163.4	1.530	31.1	29.6	-4.8†
		50.5		1.530	30.9	29.5	-4.5
JR(♀)	27	55.3	159.7	1.565	28.2	30.8	+9.2
		55.4		1.565	29.5	28.2	-4.4†
		56.4		1.580	29.6	30.5	+3.0
EL(♀)	27	51.6	166.4	1.565	29.0	31.4	+8.3†
		51.6		1.565	29.4	28.0	-4.8
		51.7		1.565	30.4	30.3	-0.3†
HD(♀)	29	50.9	166.6	1.560	32.2	33.3	+3.4
		51.1		1.560	30.5	32.2	+5.6†
AI(♀)	31	61.9	175.1	1.755	33.0	31.0	-6.1
		62.7		1.765	31.4	31.6	+0.6†
VT(♀)	31	62.4	171.0	1.730	30.4	31.3	+3.0
		62.8		1.735	30.9	30.4	-1.6†

†In these experiments the determination with the McKesson Metabolor preceded the observation with the chamber procedure.

TABLE II—CONT'D

SUBJECT, SEX	AGE (YEARS)	WEIGHT (KG.)	HEIGHT (CM.)	BODY SURFACE (SQ. M.)	CAL./HR./SQ. M. BY CHAMBER METHOD	CAL./HR./SQ. M. BY MC KESSON METHOD	DIFFERENCE (PER CENT)
MMF(♀)	32	73.6	170.1	1.850	34.8	34.4	-1.1
		73.1		1.845	33.2	33.2	±0.0†
CW(♂)	35	68.3	172.8	1.810	36.3	36.2	-0.3†
		68.3		1.810	35.6	34.4	-3.4
TM(♂)	37	66.0	167.8	1.750	35.5	32.5	-8.4†
		65.2		1.740	34.4	34.6	+0.6
GMK(♀)	38	66.2	168.4	1.755	33.8	30.9	-8.6†
		66.6		1.760	30.5	31.0	+1.6
KW(♀)	43	74.4	157.9	1.760	32.6	34.0	+4.3†
		74.5		1.760	35.0	35.5	+1.4

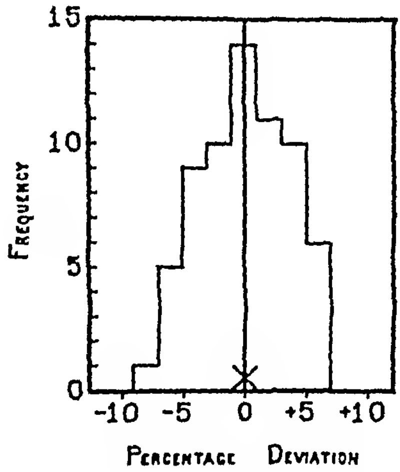
it was found that, whichever procedure was followed, the value obtained with the bedside apparatus was in some instances higher and in some lower than that procured with the chamber method. The tests in which the bedside procedure was performed first are indicated by a dagger (†) in Tables I and II.

The coefficient of correlation of the calories per hour per square meter of body surface as determined by the two methods has been calculated by the formula, $r = \dfrac{\dfrac{\Sigma xy}{n} - m_x m_y}{\sigma_x \sigma_y}$, where x represents the calories per hour per square meter as determined by the chamber method; y, the calories per hour per square meter as determined with the McKesson Metabolor; n, the number of tests; m, the mean; and σ, the standard deviation of the calories per hour per square meter. The formula, $\sigma_r = \dfrac{1 - r^2}{\sqrt{n}}$, has been used to determine the standard error of the coefficient of correlation. The value found for the coefficient of correlation of the calories per hour per square meter of body surface as determined by the two methods is 0.94 with a standard error of ±0.013 for children and 0.81 with a standard error of ±0.046 for adults. These values show an excellent correlation between the two methods both for children and for adults.

Table I shows also that reliable determinations of basal metabolism can be procured with a bedside apparatus on children under 8 years of age. This is evident, since the results of the determinations with the portable apparatus on the sixteen younger children are as satisfactory as are those on the children over 8 years old. A preliminary period of training to procure successful determinations was found necessary with three of the children between 2 and 8 years, but five of the twenty older children also required a period of training. These eight children are indicated by a double dagger (‡) in Table I. Several other investigators, who have used a mask or a mouthpiece and a nose clip, have reported satisfactory results on children under eight years old. Göttche[8] procured reliable determinations on four children and Farr[9] on seven children between 3 and 8 years of age. In the large series of de Bruin[10] there were 196

children between the ages of 1 and 8 years on whom successful observations were made. Both Farr and de Bruin reported that a preliminary period of training was frequently necessary. The results of the present study and of the other investigations cited would seem to refute the contention of Talbot, Wilson, and Worcester[11] that children of 7 years or younger cannot be tested with a portable apparatus.

The fact that satisfactory determinations of basal metabolism on children have been obtained both with the chamber procedure and with the bedside apparatus shows that factors other than the type of apparatus used determine the success to be attained when children are studied. Among these factors are those inherent in the subject on whom the determinations are being made. All workers in the field of energy metabolism realize how essential it is to obtain the cooperation of the subject to the end that he will remain quiet throughout the determinations. They will also agree that it is more difficult to secure cooperation from children, and particularly from young children, than from adults. This difficulty may be overcome, however, if the persons who conduct the test have experience with children and are patient. It is first necessary to win the child's confidence and then to enlist his help in making the determination a success. When full cooperation is procured in this way, a satisfactory determination of basal metabolism can be obtained.



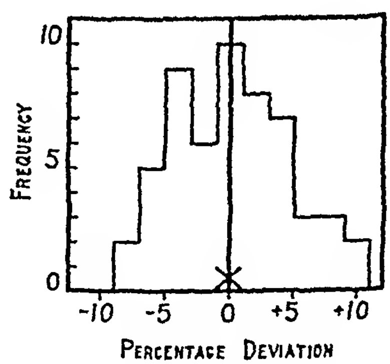


Fig. 1.—Frequency distribution of the percentage deviation of the calories per hour per square meter obtained on children with the closed circuit bedside method from those found with the open circuit chamber procedure. X represents the mean percentage deviation with regard to sign.

Fig. 2.—Frequency distribution of the percentage deviation of the calories per hour per square meter obtained on adults with the closed circuit bedside method from those found with the open circuit chamber procedure. X represents the mean percentage deviation with regard to sign.

Reference has been made in the beginning of this report to the standards of basal metabolism established by the investigations of the Child Research Council at Denver. These standards which were first published by Lewis, Kinsman, and Iliff[1] in 1937 have been slightly modified by the inclusion of data obtained since that time, and may be found with tentative standards for the fourteenth, fifteenth, and sixteenth years in a forthcoming article by Lewis, Duval, and Iliff.[12] From the results of the present study it may be concluded that these

standards for basal metabolism of children are equally reliable whether a closed circuit bedside method or a chamber procedure is used.

SUMMARY

1. Either immediately preceding or immediately following a determination of basal metabolism with an open circuit chamber method, 65 determinations on 36 children between the ages of 2 and 16 years and 55 determinations on 25 adults were made with a closed circuit bedside apparatus.

2. An excellent correlation between the results obtained by the two methods was found both for children and for adults.

3. Evidence is presented that, contrary to general belief, reliable determinations of basal metabolism on children under 8 years of age may be procured with a bedside type of apparatus.

4. The importance of obtaining cooperation from children in order to insure the successful determination of basal metabolism and the means employed to accomplish this end are discussed.

5. The standards for the basal metabolism of children established by the Child Research Council of Denver with an open circuit chamber procedure are shown to be suitable for use when the determination is made with a portable closed circuit apparatus.

REFERENCES

1. Lewis, Robert C., Kinsman, Gladys M., and Iliff, Alberta: The Basal Metabolism of Normal Boys and Girls From Two to Twelve Years Old, Inclusive, Am. J. Dis. Child. 53: 348, 1937.
2. Peterson, Anne, and Walter, Will: Basal Metabolism and Ideal Weight and Pulse Ratios as Shown by the Findings in More Than Twenty-Five Hundred Observations on About Twelve Hundred Subjects: A Preliminary Report, J. A. M. A. 78: 341, 1922.
3. Hunt, T. C.: Determination of the Basal Metabolic Rate, Lancet 1: 172, 1926.
4. Robertson, J. Douglas: A Comparison Between the Open- and Closed-Circuit Methods for Determining the Basal Metabolism, Lancet 2: 815, 1937.
5. Michaelis, A. M.: Clinical Calorimetry XXXVI. A Graphic Method of Determining Certain Numerical Factors in Metabolism, J. Biol. Chem. 59: 51, 1924.
6. Lewis, Robert C., Duval, Anna Marie, and Iliff, Alberta: A Further Study of the Basal Metabolism of Normal Boys and Girls From Two to Twelve Years Old, Inclusive, Am. J. Dis. Child. Accepted for publication.
7. Jones, Hörry M.: An Accuracy Check for Metabolism Apparatus, J. Lab. & Clin. Med. 14: 750, 1929.
8. Göttche, Oskar: Gasstoffwechseluntersuchungen im Kindesalter. Die Pubertätsreaktion, Monatschr. f. Kinderh. 32: 22, 1926.
9. Farr, Lee E.: Basal Metabolic Rate of Young Children With Nephrotic Syndrome, Am. J. Dis. Child. 56: 309, 1938.
10. de Bruin, M.: Basal Metabolic Rate in Children With Abnormal Bodily Dimensions, Am. J. Dis. Child. 57: 29, 1939.
11. Talbot, Fritz B., Wilson, Edwin B., and Worcester, Jane: Basal Metabolism of Girls: Physiologic Background and Application of Standards, Am. J. Dis. Child. 53: 273, 1937.
12. Lewis, Robert C., Duval, Anna Marie, and Iliff, Alberta: Standards for the Basal Metabolism of Children From Two to Fifteen Years Old, Inclusive. To be published.

LABORATORY METHODS

GENERAL

BIOPSY OF BONE MARROW PERFORMED BY A NEW AND SIMPLE INSTRUMENT*

HENRY TURKEL, M.D., DETROIT, MICH., AND
FRANK H. BETHELL, M.D., ANN ARBOR, MICH.

THE examination of marrow usually obtained from the sternum by aspiration has become an almost universal practice in the diagnosis of hemopoietic disorders, disturbances of the reticulo-endothelial system, certain infectious diseases, and primary or metastatic neoplasms within the marrow cavity. The advantages of the aspiration method lie in the ease in which the test may be performed, its relative freedom from discomfort or inconvenience to the patient, and the technical similarities between the stained films of aspirated marrow and those prepared from circulating blood. On the other hand, the aspirated material may not be representative of the quantitative relationship between cellular elements within the marrow; in myelosclerosis, no material may be obtainable; and in hypoplastic states, the information gained by aspiration is of uncertain significance. Moreover, films prepared from the mixture of marrow and blood secured by aspiration permit no conclusions regarding the anatomical relationship of the marrow elements.

The limitations of the aspiration method of sternal biopsy have led many hematologists to place chief reliance upon the examination of material obtained by trephine. A number of instruments have been derived for this purpose and that employed by Dameshek[1] is particularly satisfactory. Nevertheless, the minor surgical procedure involved, with incision and resulting scar, has precluded the general application of the method for diagnostic purposes.

It is the object of this communication to describe an instrument designed to obtain a small specimen of marrow with no more effort and actually less discomfort to the patient than are involved in the ordinary aspiration procedure. This device, with minor modifications, is also adapted to the securing of biopsy material from tumors and soft tissues. This instrument consists of two needles with stylets, an outer guiding needle of 14 gauge (*A*), and an inner trephine needle of 17 gauge (*B*), Fig. 1.

*From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan.

Received for publication, Sept. 4, 1942.

The external guiding needle should be 2 to 3 cm. in length. One end of this needle has a sharp beveled point, while the other end has a hollow head with a slot into which the projection of the head of the stylet fits. This arrangement keeps the point of the needle and the point of the stylet at the same cutting angle. The purpose of the guiding needle is to cut (split) through the skin and subcutaneous tissue without excising them, and to direct the inner trephine needle to the desired position.

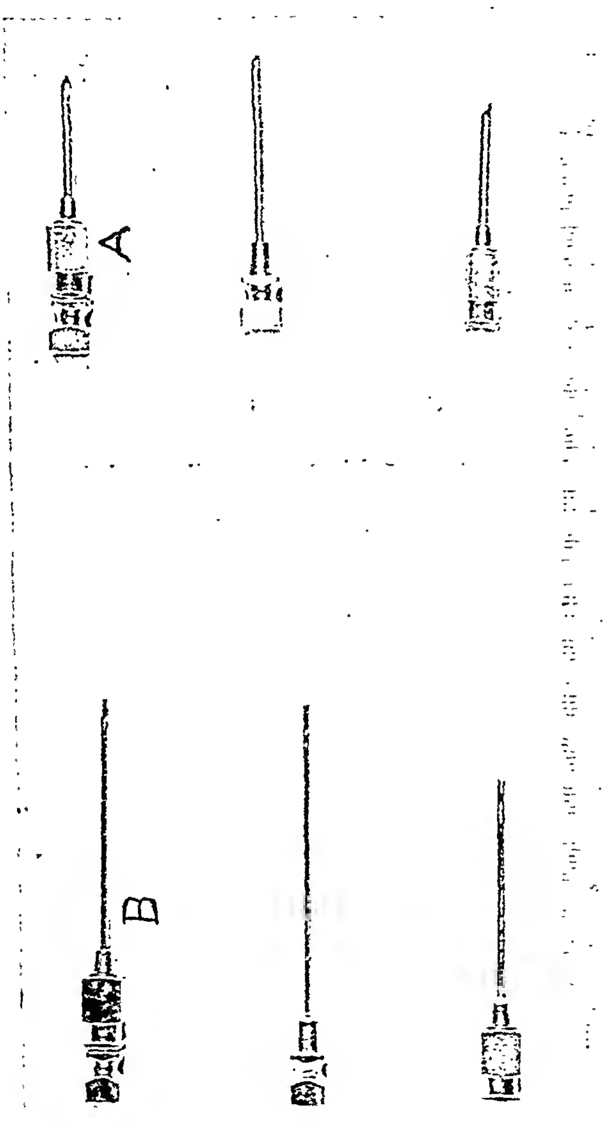


Fig. 1.—Trephine needle for bone marrow biopsy.

The internal or cutting needle, whose diameter is equal to that of the stylet of the external needle, is designed to be slipped within the external needle after the stylet is removed. The length of the two needles may be varied, but the relation must be such that the shaft of the internal needle is 5 mm. longer than the entire length of the external needle. The tip of the inner needle possesses four

saw teeth. The inside surface of the tip is cone-shaped or converging, so that the tissue becomes wedged into the needle (like a cork stopper into a flask) after being cut, and remains within the needle when the latter is removed.

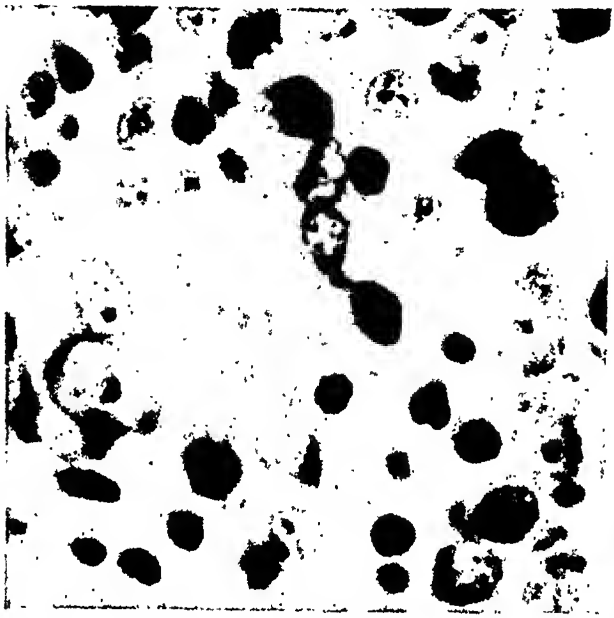


Fig. 2.—Marrow section, physician 57 years of age, suffering with subleucemic myeloblastic leucemia. No diagnostic cells were found in the blood film or aspirated material. Subsequently the disease entered a leucemic phase.

Fig. 3.—Marrow section, female 57 years of age, exhibiting nests of carcinoma cells not demonstrable by the aspiration technique. The patient was suffering with carcinoma of the breast.

The head of the internal needle is hollow, but without a slot. A standard adapter or syringe would fit into the heads of both needles. The stylet has no

projection on its head, but its stem is slightly longer than the inner needle, thus extending through the tip. This additional length serves to express the biopsy

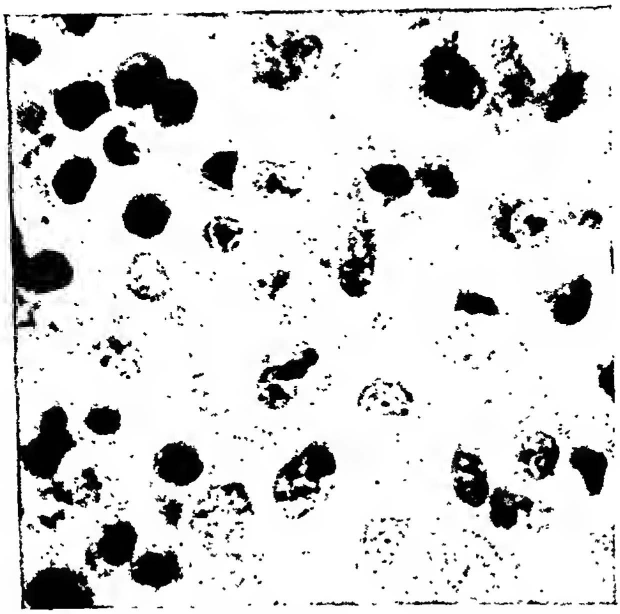


Fig. 4.—Marrow section, 44-year-old male, with multiple myeloma. Atypical plasma cells and plasmablasts were also seen in the films of aspirated marrow.

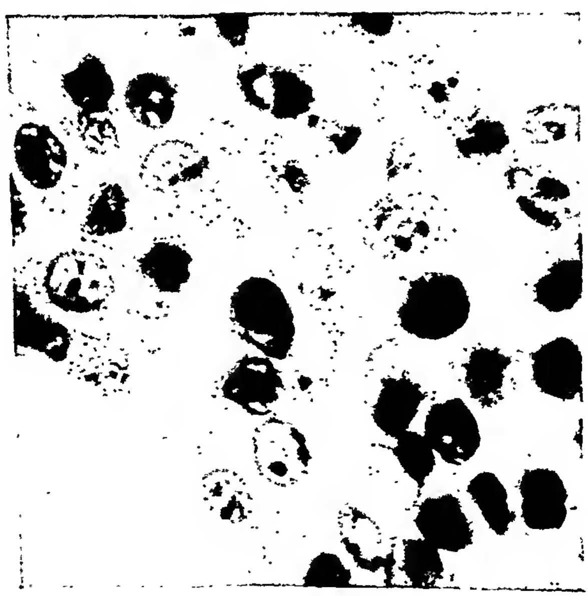


Fig. 5.—Marrow section, female 65 years of age, with subleucemic reticulosis (histiomonocytic leucemia). In this case the aspiration films were diagnostic.

material out of the inner needle and also protects the cutting tip from injury when the inner needle is inserted into the outer needle.

TECHNIQUE FOR STERNAL TISSUE BIOPSY

1. Cleanse the skin over the desired site, usually opposite the third rib, with a surgical antiseptic solution.
2. Infiltrate the skin, subcutaneous tissue, and periosteum with a local anesthetic (procaine 1.5 per cent).
3. Insert outer needle with stylet in place, with the tip of needle in direction of the face of patient at an angle of about 45 degrees, through the skin, subcutaneous tissue, and periosteum, until point just engages the anterior lamella of the sternum.
4. Remove stylet of the outer needle.
5. Insert cutting needle with stylet into outer needle.
6. Remove stylet of inner needle.
7. While holding the head of the outer needle with fingers of the left hand, turn the head of the cutting needle with fingers of the right hand backwards and forwards through a half circle, at the same time exerting gentle pressure. A sudden release of resistance signifies the entrance of the cutting tip into the sternal cavity. The cutting action with slight pressure should be continued until the needle has entered the sternal cavity for a distance of 2 to 3 mm. The needle should then be revolved several times without further insertion in order to insure detachment of the plug from the surrounding marrow.
9. Remove the inner needle.
10. Remove the outer needle.
11. Cover the point of insertion with a collodion dressing.
12. Insert stylet into the inner needle and push out the biopsy core into a bottle containing fixative solution.

It is usually also desirable to prepare films of aspirated marrow and for this purpose material obtained from the trephine hole is not, as a rule, satisfactory, because of the admixture of excessive amounts of blood. We have preferred to anesthetize a sufficiently large area to permit insertion of an aspiration needle about 1 cm. below the point of introduction of the trephine. The additional manipulation causes the patient no further discomfort, and in fact, he is usually not aware that two punctures have been made.

For fixation we have employed 100 c.c. of Zenker's solution with 5 c.c. of glacial acetic acid added just before using. After twenty-four hours, no further decalcification is required. The specimens are then washed, dehydrated, cleared, and imbedded in paraffin in the usual manner. The sections are cut with a thickness of five microns. The most satisfactory stains which we have used are Giemsa-Wolbach[2] and Kingsley.[3]

The procedure has been carried out, in conjunction with aspiration, on one hundred twelve patients representing a wide variety of hematologic conditions, and suspected primary or metastatic neoplasms of the marrow. A detailed analysis of the observations made on these patients, as well as a much larger series in which aspiration alone was done, will be published in a later communication.

Only rarely is positive diagnostic information obtained by the trephine method and not by aspiration, but such cases do occur. (Figs. 2 and 3.) The

accompanying photomicrographs were made with a 2 mm. apochromatic objective and 10× compensating ocular.

SUMMARY

1. The advantages and limitations of the aspiration method of marrow biopsy are discussed.

2. An instrument is described which enables a small specimen of marrow to be obtained by trephine without a skin incision. Photomicrographs of marrow sections secured by this method are presented.

REFERENCES

1. Dameshek, W.: Biopsy of the Sternal Bone Marrow, Am. J. M. Sc. 190: 617, 1935.
2. Wolbach, S. B.: Studies on Rocky Mountain Spotted Fever. J. M. Research 41: 1, 1919.
3. Kingsley, D. M.: A New Hematological Stain, Stain Technol. 10: 127, 1935.

THE NONSPECIFICITY OF THE SERUM-OPACITY TEST FOR CLOSTRIDIUM WELCHII*

LYLE A. WEED, PH.D., M.D., AND SHERMAN MINTON, JR., M.D.
INDIANAPOLIS, INDIANA

INTRODUCTION

THERE has long been a need for the rapid identification of *Clostridium welchii* in specimens taken from traumatic cases with symptoms and signs of gas gangrene. In time of war this need is much greater and the available laboratory facilities are usually fewer. It is quite natural therefore in the present emergency to attempt to utilize the reaction described by Nagler,[1] since it is supposed to be specific for the Type A toxin of *Cl. welchii.* It is the purpose of this communication to call attention to the error of using such a test, which in reality is entirely nonspecific; because other antisera will prevent the reaction, and many common aerobes ordinarily present in contaminated wounds may also give a positive reaction.

HISTORICAL

In 1939 Nagler[1] pointed out that *Clostridium welchii* grown in a mixture of human serum and broth produced opacity in the medium. Usually, but not always, a curd was formed which on centrifuging rose to the surface of the fluid, while the bacterial cells deposited on the bottom of the tube left a clear fluid intervening. This reaction could be entirely prevented by growing the organisms in a similar mixture which contained in addition *Cl. welchii* antitoxin. All strains of *Cl. welchii* tested gave the reaction, but all other anaerobes used did not. These facts suggested the reaction was specific for *Cl. welchii* and that it was probably due to Type A toxin, since all four types (A, B, C, and D of Wilsdon's classification[2]) gave the reaction.

*From the Department of Bacteriology and the Research Division, Indiana University Medical Center, Indianapolis.

Received for publication, August 15, 1942.

Recently Hayward[3] reported the use of this reaction in the rapid identification of *Clostridium welchii* in clinical material submitted for examination. She also tested 174 strains of known non-welchii anaerobes and found that 9 gave positive results or 5.1 per cent. She also found considerable variation in the amount of inoculum required to obtain a positive reaction when using known strains of *Cl. welchii*, the required number of organisms varying from as low as 400 to as high as 200,000. Whereas Nagler originally found the reactions to be positive in 16 to 18 hours' cultivation, she found some strains required as much as three days to produce opacity or coagulation. There is no evidence that she tested any of the common aerobes which would likely be present in clinical material submitted for routine bacteriologic examination. To date we have found no literature indicating any one has studied this reaction employing aerobes in place of anaerobes except the report by Nagler,[1] who used a pseudoanthrax organism.

TABLE I

PRODUCTION OF SERUM OPACITY BY VARIOUS ORGANISMS AND THE INHIBITION OF THE REACTION BY USE OF NONSPECIFIC ANTITOXINS

ORGANISMS		ANTISERUM USED				
		NONE	*Cl. welchii*	*Cl. tetani*	*Vib. septique*	*C. diph.*
Cl. welchii	H	+	-	-	-	
	M	+	-	-	-	
	C	+	-	-	+	
	G	+	+	-	+	
	3628	+	-	-	-	
	3630	+	+	-	+	
	3631	+	-	-	+	
	3626	+	+	+	+	
	3627	+	+	+	+	
	DR 2	+	-	-	+	
	SR_2 2	+	-	-	+	
	6504	+	-	-	-	
	6506	+	-	-	-	
	6509	+	+	+	+	
	7907	+	-	-	-	
Cl. novyi	7658	+	-	-	-	
Cl. putrificum	4996	+	-	-	-	
Cl. botulinum	687	+	-	-	-	
Cl. botulinum	6060	+	-	-	-	
Cl. bifermentans	638	+	-	-	+	
Cl. oedematis maligni	461	+	-	-	-	
Proteus vulgaris		+	-	-	-	-
Escherichia coli	5	+	-	-	-	-
Escherichia coli	105	+	+	-	-	-
Aerobacter aerogenes		+	-	-	-	-
Pseudomonas aeruginosa		+	-	-	-	-
Diphtheroid	R 1194	+	+	-	-	-
Staphylococcus aureus	S	+	-	-	-	-
	PLR 1	+	-	-	-	-
	PLR 2	+	-	+	+	-
	a	+	-	-	-	-
	b	+	+	-	-	-
	R 1216	+	+	-	-	-
	KH 2	+	-	-	-	-
	KH 3	+	-	-	-	-
Staphylococcus albus	K 3	+	-	-	-	-
	S	+	-	-	-	-
	C	+	-	-	-	-
Staphylococcus citreus		+	-	-	-	-

EXPERIMENTAL

The technique described by Nagler was followed in detail except where other antiserums were used in place of *Cl. welchii* antitoxin. The tubes were incubated eighteen hours in the anaerobic jar. The controls always showed good growth and the reactions were definite.

Table I shows the production of a positive reaction with many aerobes and anaerobes other than *Cl. welchii* and the inhibition of the reaction with various types of antitoxins. It also shows the inability of *Cl. welchii* antitoxin to prevent the reaction. The data very clearly demonstrate the nonspecificity of the reaction.

CONCLUSION

The serum opacity reaction for the identification of *Cl. welchii* may be prevented by heterologous antitoxins. The reaction is produced by many common aerobes and anaerobes and these reactions may likewise be prevented by the use of nonspecific antitoxins. The reaction should not be used for the identification of *Cl. welchii.*

REFERENCES

1. Nagler, F. P. O.: Observations on a Reaction Between the Lethal Toxin of Cl. Welchii (Type A) and Human Serum, Brit. J. Exper. Path. 20: 473, 1939.
2. Wilsdon, A. J.: Observations on the Classification of Bacillus Welchii, University of Cambridge, Institute of Animal Pathology, Report 2: 85, 1931.
3. Hayward, Nancy J.: Rapid Identification of Cl. Welchii by the Nagler Reaction, Brit. M. J. 1: 811, 1941.

DOSING OF POWDERED DRUGS TO SMALL ANIMALS*

MAX STERNE
TRANSVAAL, SOUTH AFRICA

I HAVE noticed in several recent publications that workers use the stomach tube for dosing small animals with powders such as sulfapyridine. This is neither accurate nor easy, and is risky when animals are weak or ill as a result of experimental procedures.

The instrument described in Fig. 1 costs nothing. With it, small animals such as guinea pigs, rabbits, and cats can be dosed safely and expeditiously.

The end of the barrel of a broken-tipped 1-c.c. tuberculin syringe is cut off and ground flat. The outer edge is beveled slightly so as to avoid a cutting edge. A light metal sleeve, which can be cut from the guard in which large veterinary hypodermic needles are packed, is fitted over the plunger and adjusted so that ½ mm. of the plunger projects when this is pushed home into

*From the Onderstepoort Laboratories.
Received for publication, Sept. 10, 1942.

the barrel. The sleeve simply acts as a stop. An instrument without the sleeve, and with the plunger cut to the proper length, is not nearly so handy. The making takes about fifteen minutes.

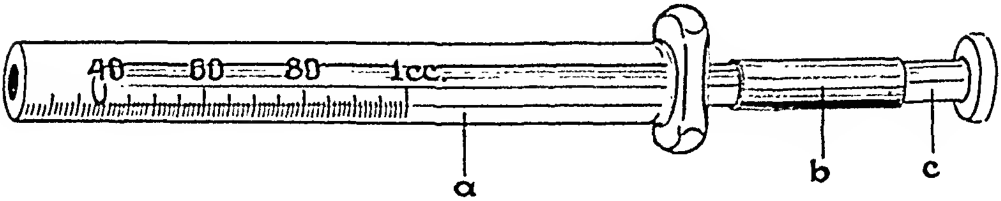


Fig. 1.—a, barrel; b, sleeve; c, plunger.

TABLE I

WEIGHTS OF TWENTY SUCCESSIVE FILLINGS

TO ADMINISTER 100 MG.	TO ADMINISTER 200 MG.
99.5	203.0
100.0	203.0
100.0	199.0
101.0	202.0
103.0	201.5
102.5	199.0
100.0	203.0
103.5	201.0
101.5	203.0
102.5	201.0

The instrument, which is merely a small balling gun, is filled by withdrawing the plunger to a mark calibrated for the required dose (one of the original gradations), and dabbing the end of the barrel into a small container of the powder until the powder reaches the end of the plunger. The end of the balling gun is pushed over the back of the animal's tongue, and the load discharged. A good degree of accuracy is obtained by this filling method. Table I gives the weights of twenty successive fillings for administering 100 and 200 mg. doses of sulfapyridine to guinea pigs.

AN IMPROVED METHOD FOR THE ISOLATION OF MANNITOL-FERMENTING SHIGELLAE*

OSCAR FELSENFELD,† M.D., MANTENO, ILL.

INDUCED by the unsatisfactory results encountered with the preserving fluids in prevalent use for Shigellae, search was instituted for more adaptable media. Among others, the fluid of Bangxang and Eliot[1] was tested for such suitability. Although announced over two years ago, this medium apparently had not obtained extensive application, and in order to ascertain its effectiveness, a test was made with laboratory strains of Shigellae, and with feces infected, in vitro, with Shigellae.

The subcultures from Bangxang and Eliot's medium inoculated in this manner proved rich in Shigellae for a period of at least four days. Because sulfa drugs may inhibit the growth of Shigellae, the fluid was tested also for its suitability in preserving Shigellae in the feces of persons treated with such drugs. The necessity to counteract sulfa drugs presented itself as an urgent task. The amount of 5 mg. p-aminobenzoic acid per 100 c.c. of the fluid was found sufficient to abolish the inhibitory effect on any microbes of sulfanilamide, sulfathiazole and sulfaguanidine when these drugs were given in the usual therapeutic concentrations. The amount of 5 mg. per 100 c.c. was chosen as a standard supplement of Bangxang and Eliot's preservative. P-aminobenzoic acid in this concentration does not stop the growth of mannitol-fermenting Shigellae.

It was found that the optimal efficacy of the preserving fluid is guaranteed at a pH around 8 and that dried bile may be substituted for sodium desoxycholate. Therefore the medium was prepared in the following modification:

Dissolve 10 Gm. of sodium citrate, 10 Gm. of peptone, 5 Gm. of sodium desoxycholate (or 6 Gm. of dried bile) and 9 Gm. of sodium chloride in 1 L. distilled water. Add 3 c.c. of a 1 per cent monosodium phosphate solution, neutralize with n/10 sodium hydroxide. Add 0.05 Gm. p-aminobenzoic acid; adjust pH to 8 with sodium hydroxide; tube, or put in containers filling up one-third of the glass. Sterilize in flowing steam.

For the diagnosis of the members of the Shigella group, plating on several media is an established routine. A variety of media was plated from the Bangxang and Eliot fluid, and the outcome evaluated.

The new plate of Wilson and Blair was used simultaneously with the plates of MacConkey; Holt, Harris, and Teague; Leifson; Endo; Wilson, Blair, Hajna and Perry; and selenite enrichment with consecutive plating on one of Leifson's plates, in the examination of proctoscopic specimens.

*From the Bacteriological Laboratory of the Manteno State Hospital, Manteno, Ill.
Received for publication, Sept. 19, 1942.
†Present address: Chicago State Hospital, Chicago, Ill.

Shortly after Wilson and Blair[2] described their new medium for the cultivation of *Shigella paradysenteriae*, it was praised by Thomas and Hulme.[3] On this plate, *Shigella paradysenteriae* grows in pink or colorless colonies. Strong lactose fermenters form a yellow growth. Escherichiae, Aerobacters, Protei, and Pseudomonades are said to be retarded.

It was found that the rosolic acid and the potassium tellurite used in the new medium of Wilson and Blair have to be standardized against known strains. The pH of the medium must be carefully observed. The alteration of the pH by the addition of the iron citrate, rosolic acid, and tellurite must be corrected to the final optimum of 7.6 to 7.8. At this pH, not only Flexner strains, but also Sonne strains grow on this medium very well. Heavy inocula may be used without courting the danger of overgrowth. While some fast growing strains of Proteus and Pseudomonas render the interpretation even of such selective plates as those of Leifson difficult, Wilson and Blair's medium is not disturbed by spreaders.

MacConkey's medium was prepared with the addition of 0.3 Gm. chloral hydrate per liter.[4] In this form, the spreading of most Proteus strains is checked. Addition of 3 c.c. of a 0.4 per cent solution of bromthymol blue per liter of medium rendered the medium more legible.

The media of Holt, Harris, and Teague, Leifson's desoxycholate plate, and Endo's medium were prepared according to the prescriptions of the American Standard Methods.[5] Leifson's desoxycholate-citrate plate was purchased on the open market. Endo's medium was omitted after it failed in 60 per cent of the first series of examinations.

Over eight hundred proctoscopic specimens were examined one to six hours after they were taken. Fifty-one specimens harbored *Shigella paradysenteriae* (Flexner). Twenty-two revealed *Shigella sonnei*, and one contained members of both groups. Many positive specimens were taken from the same individual over varying time intervals.

Twenty-five specimens gave a positive result on all four plates (desoxycholate-citrate, MacConkey, Wilson-Blair, and Teague).

Ten specimens were positive on desoxycholate-citrate, MacConkey, and Wilson-Blair medium, but negative on Teague.

Five specimens were positive on desoxycholate-citrate, Wilson-Blair, and Teague, but negative on MacConkey.

Seven specimens were positive on desoxycholate-citrate, MacConkey, and Teague, but negative on Wilson-Blair.

Two specimens were positive on MacConkey, Wilson-Blair, and Teague, but negative on desoxycholate-citrate.

Two specimens were positive only on desoxycholate-citrate and MacConkey.

Eight specimens were positive only on desoxycholate-citrate and Wilson-Blair.

Eight specimens were positive only on MacConkey and Teague.

Three specimens were positive only on desoxycholate-citrate.

One specimen was positive only on MacConkey.

Two specimens were positive only on Wilson-Blair.

One specimen was positive only on Teague.

Summarizing these results, from the seventy-four positive specimens:

Sixty were positive on the desoxycholate-citrate plate	82 per cent.
Fifty-eight were positive on MacConkey's medium	78 per cent.
Fifty-six were positive on Wilson-Blair's new plate	70 per cent.
Forty-eight were positive on Teague's medium	65 per cent.

All three forms of colonies (S, R, and G) appeared only on the plate of MacConkey, on Leifson's desoxycholate medinm, and to a smaller extent on Teague's plate. The desoxycholate-citrate medium does not permit the growth of all forms, an observation made already by Ruys.[6] Wilson and Blair's plate showed the same characteristics. The advantage of a "heavier" inoculum on selective media is therefore equilibrated by the advantage of "all colony forms growing" on nonselective plates.

Bangxang and Eliot's preserving fluid was effective in our cases when, between the taking of the specimen and the plating, some hours had elapsed.

It should be added that the procedure described revealed not only mannitol-fermenting Shigellae, but in two cases, *Eberthella typhi,* and in two other cases, *Salmonella suipestifer.*

SUMMARY

It seems that the only way to obtain a high number of positive results is to use several selective and nonselective plates simultaneously. The preserving fluid of Bangxang and Eliot is recommended. From the selective media those of Leifson, and of Wilson and Blair, from the nonselective plates those of MacConkey, and of Teague proved to be satisfactory. Wilson and Blair's new plate is not superior to other media. It is easy to prepare, it is selective, and it deserves therefore to be added to the series of routine plates.

I wish to thank Dr. H. J. Shaughnessy, Chief, Division of Laboratories, Department of Public Health, Chicago, Ill., and his assistant, Miss F. Friewer, Chief of the Typhoid Section of the Central Laboratory of the Department of Public Health, Chicago, Ill., for constant advice and helpful suggestions in this work.

REFERENCES

1. Bangxang, E., and Eliot, C. P.: An Investigation of Preserving Solutions for the Recovery of Dysentery Bacilli From Fecal Specimens, Am. J. Hyg. 31: 16, 1940.
2. Wilson, W. J., and Blair, E. M.: A Tellurite-Iron-Rosolic Acid Medium Selective for B. dysenteriae (Flexner), Brit. M. J. 2: 501, 1941.
3. Thomas, J. C. S., and Hulme, W. A.: A Tellurite Medium in the Diagnosis of Dysentery, Lancet 1: 321, 1942.
4. Felsenfeld, O., and Grant, W. H.: A Study of Methods Used for Inhibition of the Growth of Proteus on Diagnostic Media, J. Bact. 43: 23, 1942.
5. Diagnostic Procedures and Reagents, New York, 1941, American Public Health Association.
6. Ruys, A. C.: Isolation of Typhoid, Paratyphoid, and Dysentery Bacteria From Faeces and Urine, Brit. M. J. 1: 606, 1940.

A METHOD FOR THE DETERMINATION OF STAPHYLOCOCCAL ANTITOXIN AND ANTICAPSULAR AGGLUTININ USING CAPILLARY BLOOD*

JOHN A. LICHTY, JR., M.D., AND CHRIS P. KATSAMPES, M.D., ROCHESTER, N. Y.

DURING a study of staphylococcal antitoxin and anticapsular agglutinin in the blood of mothers and their infants, the difficulty of obtaining sufficiently large samples of venous blood from the infants prompted us to devise a method that would require only the small amounts of capillary blood obtainable by heel puncture. The method described herewith has proved to be highly satisfactory.

PREPARATION OF SAMPLE

Exactly 0.05 c.c. of capillary blood is drawn into a glass pipette previously rinsed with a 0.2 per cent solution of potassium oxalate in saline. The blood is introduced into a 10 mm. glass tube containing 0.45 c.c. of the oxalate solution and is shaken vigorously to avoid clotting. After centrifuging for a few minutes, 0.35 c.c. of the supernatant can be removed for the two determinations.

A. DETERMINATION OF ANTITOXIN

For titration of antitoxin, 0.25 c.c. of the supernatant is diluted in series, using 10 mm. test tubes containing 0.25 c.c. of physiologic saline (0.85 per cent NaCl). To each tube is added 0.25 c.c. of a 1:4 dilution of the staphylococcal toxin (hemolysin), as used for the routine titration of venous serum antitoxin, and the mixture is placed in a water bath at 37° C. for fifteen minutes. Finally, 0.5 c.c. of a 1 per cent suspension of fresh, thrice-washed rabbit erythrocytes is added to each tube, bringing the total volume to 1 c.c. The tubes are again placed in the water bath at 37° C. for an hour and then left in an icebox at 4° C. overnight. The following day the test mixtures are kept at room temperature for ten minutes before the presence of hemolysis is observed by gently rotating each tube in the rack. The hemoglobin always rises from the bottom of the tube before the unhemolyzed cells. In a dilution series the last tube showing no hemolysis represents the end point.

For each group of microdeterminations two controls were always used: (1) titration of a standard staphylococcal antitoxin by the routine method; (2) simultaneous titration of one subject's antitoxin by the routine, venous serum method and the micromethod. The standard antitoxin, a commercial preparation obtained from The Lederle Company, Pearl River, N. Y., was adjusted by dilution with saline so that 1 c.c. contained exactly one international unit of staphylococcal antitoxin. This preparation was stored in a sealed flask at 4° C. For the routine titration of antitoxin in venous serum, the method of

*From the Departments of Pediatrics and Bacteriology, University of Rochester School of Medicine and Dentistry and Strong Memorial and Rochester Municipal Hospitals.

Received for publication, September 24, 1942.

Parish et al.[1] was used. The staphylococcal toxin was prepared in a manner similar to that of Dolman.[2] Complete details of these two procedures are presented elsewhere.[3]

A sample group of titrations is shown in Table I. By including the titration of a standard antitoxin, the use of any convenient strength (X) of toxin is possible, and the antitoxic titer of venous serum for Subject A can readily be expressed as international units. The 1:4 (X/4) dilution of toxin used in the micromethod was obtained empirically so that the end point for this method would be at the same tube as that for the routine method. For Subject A the end point for both methods is at Tube 4. It must be emphasized that the actual amount of serum in each dilution tube of the micromethod is not the same as that for the corresponding tube of the routine method. If one can standardize the micromethod so that the values obtained by it give a constant ratio with those of the routine method, however, the amount of antitoxin present in the sample of capillary blood can be expressed in terms of international units per c.c. of serum. This is indicated in Table I by the values in parentheses.

TABLE I

SAMPLE TITRATION OF STAPHYLOCOCCAL ANTITOXIN BY ROUTINE AND MICRO-METHODS

SPECIMENS TESTED	METHOD	TITRATION TUBES 1	2	3	4	5	6	7	8	INTERNATIONAL UNITS OF ANTITOXIN/C.C. SERUM	STRENGTH OF TOXIN USED
Standard antitoxin (1 int. unit/c.c.)	Routine	O	O	O	O	O	H	H	H	1.0 (known)	X
Venous serum of Subject A	Routine	O	O	O	O	H	H	H	H	0.5	X
Capillary blood of A	Micro	O	O	O	O	H	H	H	H	(0.5)	X/4
Capillary blood of B	Micro	O	O	O	O	O	H	H	H	(1.0)	X/4
Capillary blood of C	Micro	O	O	H	H	H	H	H	H	(0.13)	X/4
Capillary blood of D	Micro	O	O	O	O	O	O	H	H	(2.0)	X/4

H = Hemolysis of rabbit R.B.C.
O = No hemolysis of rabbit R.B.C.
X = Any convenient, freshly prepared dilution of the concentrated staphylococcal toxin.
( ) = Explained in text.

The simultaneous determination of antitoxin by both methods in a group of twenty-six subjects indicates that the micromethod is well standardized and reliable. The results are compared in Table II. In twenty-two of the twenty-eight sets of simultaneous determinations, the end point by the two methods was exactly the same, and in four more the difference was only a single tube. This is within the probable error of any duplicate neutralization titration. Poor agreement of the two methods was obtained in only two instances. One seems justified, therefore, in concluding that the method of measuring staphylococcal antitoxin by using capillary blood will yield results comparable to those of the routine method requiring venous blood in most instances.

B. DETERMINATION OF STAPHYLOCOCCAL ANTICAPSULAR AGGLUTININ

The agglutination tests carried out in the present study followed essentially the method described by Lyons.[4] A hemolytic toxin-producing *Staphylococcus aureus* isolated from a case of acute osteomyelitis was used.

A shallow layer of Douglas's broth containing 0.05 per cent of glucose was inoculated with 0.1 c.c. of a twelve-hour culture of the organism for each 5 c.c.

of broth. After three hours' growth at 37° C., the culture was killed by slowly heating to 100° C., centrifuged and resuspended in saline to 1/20 of its original volume. This suspension was used in the agglutination.

For agglutinin titration 0.1 c.c. of the clear supernatant from the capillary blood sample was diluted with 0.7 c.c. of saline. Further dilutions were made in series by transferring 0.4 c.c. of the preceding dilution to 0.4 c.c. of saline in the next tube.

TABLE II

SIMULTANEOUS DETERMINATION OF STAPHYLOCOCCAL ANTITOXIN BY ROUTINE METHOD AND MICROMETHOD

SUBJECT	END-POINT OF ROUTINE METHOD	END POINT OF MICROMETHOD	ERROR
	Tube No.	Tube No.	
G	7	7	0
P	4	4	0
Be	5	5	0
W	2	2	0
de P	7	7	0
Do	7	7	0
H	3	4	+1 tube
Ba	2	2	0
Da	3	4	+1 tube
Li	5	5	0
	(duplicate determination)	5	
Li	5	5	0
K	5	5	0
Mo	1	1	0
La	2	2	0
Ma	3	5	+2 tubes
Mi	3	3	0
I	1	2	+1 tube
O	3	3	0
A	2	<1	-2 tubes
Ho	2	2	0
Lib	3	4	+1 tube
Oc	3	3*	0
Ca	4	4*	0
Za	5	5*	0
Mor	5	5*	0
Ba	5	5*	0
An	6	6*	0

*These tests were performed with toxin which was six months old and it was necessary to allow the tubes to stand at room temperature for one to two hours after removing them from the icebox before satisfactory hemolysis could be observed in the micromethod.

One drop of the suspension of staphylococci was mixed with a loopful of the serum dilution on a clear glass slide. A control test of the organisms and saline was always made on the same slide. The test mixtures were gently rotated for five minutes and then read. The photographic charts of Pauli and Coburn[5] served as standards for reading the degree of agglutination.

The accuracy of the method using capillary blood in the titration of the anticapsular agglutinin was determined by the simultaneous assay of venous blood serum and capillary blood. The results of seventeen parallel determinations (Table III) showed exact agreement in eleven cases (65 per cent). In the remaining six cases (35 per cent), the variation was less than one serum dilution interval.

The values used for the micromethod in Table III are based on the assumption that the supernatant of the centrifuged capillary blood represents a 1:10

TABLE III

THE SIMULTANEOUS DETERMINATION OF STAPHYLOCOCCAL ANTICAPSULAR AGGLUTININ BY ROUTINE METHOD AND MICROMETHOD

SUBJECT	SERUM FROM VENOUS BLOOD		SERUM FROM CAPILLARY BLOOD	
	COMPLETE AGGLUTINATION	PARTIAL AGGLUTINATION	COMPLETE AGGLUTINATION	PARTIAL AGGLUTINATION
J. A. L.	640	1280	640	1280
C. P. K.	640	1280	640	1280
O.	640	-	320	640
C.	640	1280	640	1280
Z.	640	1280	640	1280
J. A. L.	1280	-	640	1280
C. P. K.	640	1280	320	640
W. L. B.	640	-	320	640
A.	640	1280	640	1280
J. A. L.	640	-	640	1280
C. P. K.	1280	-	640	1280
W.	640	-	640	-
J. A. L.	640	-	640	-
B.	160	320	160	-
Mc	320	640	320	640
S.	320	640	320	640
D.	160	320	320	-

Complete agglutination = 4+
Partial agglutination = 1 to 3+
No agglutination = -

dilution of the capillary serum. This dilution is probably not 1:10, but ranges from 1:10 to 1:20, depending on the hematocrit value of the capillary blood. The results for the venous and capillary sera in Table III, however, agree more closely if the original dilution of the capillary serum is assumed to be 1:10 rather than 1:20. We have no definite explanation for this finding, but it may be due to a greater concentration of agglutinin in capillary as compared with venous serum. This notion is supported by the results obtained by calculating the ratio of staphylococcal toxin to antitoxin in the micromethod on the basis of that observed for the routine (venous serum) method. If this be done, one finds that capillary serum actually neutralizes from 2½ to 5 times the expected amount of toxin, depending on the dilution of the serum (from 1:10 to 1:20) in the sample of capillary blood. Thus, the staphylococcal antitoxin likewise appears to be more concentrated in capillary than in venous serum.

CONCLUSIONS

A method is described for the simultaneous titration of staphylococcal antitoxin and anticapsular agglutinin using 0.05 c.c. of capillary blood. A comparison of this method with the standard procedure for the titration of each antibody in venous serum shows an agreement satisfactory for serologic methods.

REFERENCES

1. Parish, H. J., O'Meara, R. A., and Clark, W. H.: The Clinical Investigation of Staphylococcal Toxin, Toxoid and Antitoxin, Lancet 1: 1054, 1934.
2. Dolman, C. E.: Pathogenic and Antigenic Properties of Staphylococcus Toxin, Canad. Pub. Health J. 23: 125, 1932.
3. Lichty, J. A., Jr., Katsampes, C. P., and Baum, W. S.: A Study of Humoral Antibodies for Staphylococcus Aureus in Infants and Their Mothers, J. Pediat. 22: 549, 1943.
4. Lyons, C.: Antibacterial Immunity to Staphylococcus Pyogenes, Brit. J. Exper. Path. 18: 411, 1937.
5. Pauli, R. H., Coburn, A. F.: Studies on Serological Typing of Streptococcus Hemolyticus, J. Exper. Med. 65: 595, 1937.

CHEMICAL

THE DETERMINATION OF THIAMIN IN BLOOD*

THEODORE E. FRIEDEMANN, PH.D., AND THADDEUS C. KMIECIAK, B.S.,
CHICAGO, ILL.

IT IS now generally agreed that the determination of thiamin in blood and urine is a valuable aid in the diagnosis of thiamin deficiency. Fairly accurate chemical methods have been described for the determination of thiamin in urine. It has been shown that the daily excretion of the vitamin by the kidney is related to the intake and absorption of dietary thiamin and that the determination is of considerable value, provided the subject continues his customary diet during the 24-hour period of urine collection.[1, 2] On the other hand, because of technical difficulties, chemical methods have not been successfully applied to blood. The most reliable results have been obtained by means of the cocarboxylase methods of Ochoa and Peters[3] and Goodhart and Sinclair[4, 5, 6] and the yeast fermentation method of Schultz, Atkin, and Frey.[7, 8] The first is specific for diphosphothiamin, or cocarboxylase; the second is not. Both are subject to many variations, and the techniques must therefore be carefully controlled. The fermentation method can be expected to yield higher results, since it determines the total thiamin content. The difference should be slight, however, because blood contains very little free thiamin. By using these procedures, Goodhart has shown that the whole blood of human subjects contains from 3 to 9 micrograms of total thiamin per 100 c.c. Thus, by means of the cocarboxylase method, this author found from 2.9 to 9.4, with an average of 5.4 micrograms per 100 c.c. in the blood of 50 children varying in age from 4 to 15 years.[9] The blood of 26 healthy adults contained from 3.2 to 8.7, with an average of 5.0 micrograms per 100 c.c.[4] When the fermentation method was used, the blood of 45 normal subjects contained from 3.1 to 9.2, with an average of 5.39 micrograms.[8] Our experience, in agreement with that of others, has shown that the total thiamin content of blood does not fluctuate as widely as the urinary excretion: it is not as rapidly affected by the dietary intake of thiamin. Yet a very definite correlation is noted between the concentration of total thiamin in the blood and the incidence of deficiency symptoms. Since a high percentage of subjects with polyneuropathy have blood values below 3 micrograms per 100 c.c., Goodhart has suggested that a concentration below this level indicates a state of deficiency.

The chemical procedure described by us, besides having improvements in technique, embodies many of the features described in the numerous modifications of the Jansen thiochrome method.[10-20] The principal difficulty in the analysis

*From the Department of Physiology of the Northwestern University Medical School and Passavant Memorial Hospital, Chicago.

This investigation was supported by a grant from the Abbott Fund of Northwestern University.

Received for publication, December 31, 1942.

of blood is in the precipitation of the large amount of soluble protein. This is readily accomplished by means of metaphosphoric acid, which always yields solutions which rapidly pass through the zeolite adsorbent and allow an excellent recovery of added thiamin. With a similar method, using trichloroacetic acid, Hennessy and Cerecedo[16] found from 9 to 12 micrograms per 100 c.c. of thiamin in blood. Our results, obtained by the analysis of 75 samples from 36 subjects, varied from 3.0 to 11.2, with an average of 5.70 micrograms per 100 c.c. Our data, therefore, agree substantially with those of Goodhart.

PROCEDURE

Reagents

Approximately N HCl and M $NaHCO_3$ Solutions

Metaphosphoric Acid.—A 10 per cent solution is prepared at frequent intervals, preferably each day. Since the acid deteriorates rapidly at room temperature, it should be dissolved in cold water in the refrigerator and the solution kept there when not in use.

Phosphatase.—A 10 per cent solution of taka diastase[16] or clarase[19] is prepared each day. Only the clear supernatant solution or filtrate is used.

Ion Exchange Adsorbents.—Artificial zeolites, such as Decalso,*[16] Permutit,*[18] or Crystallite† may be used. We prefer a mixture of equal parts of zeolites of 30- to 50-mesh and 50- to 80-mesh size. Columns prepared from such a mixture are not readily clogged with colloidal material and solid particles which sometimes are present in the final solution. One kilo of zeolite is covered and thoroughly mixed with 3 to 4 changes of an acidified (2 c.c. of concentrated HCl per liter) 25 per cent solution of NaCl. If ordinary table salt is used, the solution should be filtered before the addition of the acid. The supernatant solution is removed by decantation each time. The zeolite is then washed 5 or more times by decantation with acidified distilled water (1 c.c. of glacial acetic acid per liter). This washing removes the finer material as well as the excess of salt. The preparation is then transferred to a large Buchner funnel, is well washed with distilled water, and finally dried at room temperature in a shallow pan. In order to have the material in its maximum expanded state, a small portion is kept moistened in a widemouthed bottle.

NaCl Solution.—Each liter contains 250 Gm. of C.P. NaCl and 2 c.c. of concentrated HCl. Solutions prepared from table salt contain materials which slightly reduce the yield of thiochrome from thiamin. If table salt is used, the solution should be filtered before acidification with HCl.

Oxidizing Reagent.[11, 13, 18]—A mixture consisting of 9 parts of 10 N NaOH and 1 part of a 1 per cent solution of $K_3Fe(CN)_6$ is freshly prepared each day.

Iso-butyl Alcohol.[13]—The fluorescence without oxidation should be not greater than that of distilled water. Higher fluorescing batches may be used in the analysis of urine, but *they should never be used in the analysis of blood.* Should the alcohol contain much fluorescing material, it is acidified with con-

*This is obtained from the Permutit Company, New York City, New York.

†Crystallite, when prepared with the recommended very acid salt solution, does not adsorb the vitamin as well as Decalso. It may be obtained from the International Filter Company, 325 W. 25th Place, Chicago, Illinois.

centrated HCl, dehydrated with Na_2SO_4, and redistilled. Butyl alcohol may also be used.[12, 19]

The used alcohol is poured into a bottle which contains an excess of HCl. The acidified alcohol is distilled. Much water separates from the fraction which distills up to 100° C. All fractions contain water, which is removed with anhydrous Na_2SO_4 before redistillation. Calcium oxide is not used because traces of alkali in the distillate catalyze the formation of fluorescing substances.

Quinine Sulfate Standard.[20]—One hundred mg. of quinine sulfate, U.S.P., are transferred to a volumetric flask of 1 liter capacity. One hundred c.c. of approximately N H_2SO_4 are added to about 800 c.c. of solution and the volume is brought to the mark. The intermediate standard is prepared by diluting 25 c.c. of the stock standard to 100 c.c. with water and enough N H_2SO_4 to bring the final acidity to 0.1 N. The solutions are stable for at least one year if kept in the refrigerator in an amber-colored bottle. To prepare the working standard, exactly 5 c.c. of intermediate standard are diluted to 500 c.c. with water and H_2SO_4 as before. The solution should be prepared frequently. It should be protected from light and stored in the refrigerator. The fluorescence of 10 c.c. of this solution in a cuvette is approximately the same as that given by 10 c.c. of extract obtained after oxidation of 1 microgram of thiamin to thiochrome.

Thiamin Standard.—Exactly 50 mg. of anhydrous thiamin hydrochloride (dried several weeks over H_2SO_4) are dissolved in a 25 per cent solution of ethyl alcohol in approximately 0.01 N HCl. When brought to a volume of 500 c.c. with this solution, each c.c. contains 100 micrograms. This stock solution, if kept cold, is stable for at least six months. To prepare the intermediate standard, 25 c.c. of the stock solution are pipetted into a 250 c.c. volumetric flask which contains 100 c.c. of water and 25 c.c. of 0.1 N HCl. The volume is then brought to the mark. The working standard is prepared when needed by diluting 5 c.c. of the intermediate standard to a volume of 250 c.c. with 0.01 N HCl. Five c.c. contain 1 microgram of thiamin.

ANALYSIS OF WHOLE BLOOD

Collection of Sample.—Blood is collected with a syringe and prevented from clotting with a minimum of oxalate. *It is then stored in the refrigerator and the analysis is made within a few hours of collection.* These precautions are necessary, since the thiamin content changes gradually even on storage of the sample in the cold.

Digestion With Phosphatase.—Five c.c. of blood are mixed with 25 c.c. of water in a large heavy-walled test tube such as is used in the determination of nonprotein nitrogen. Two c.c. of N HCl are added, and the contents are mixed by means of a long thin footed glass rod.* The mixture is heated ten minutes with frequent stirring in a boiling water bath. It is then cooled, after which 1 drop of caprylic alcohol and 2 c.c. of M $NaHCO_3$ solution are added. The latter should be run in slowly and with stirring. This volume of solution is sufficient to bring the reaction to pH 4.5 to 5.5. 2 c.c. of phosphatase solution is now

*A J-shaped rod, with a round bend fitting the bottom of the tube is recommended. With such a rod the contents of the tube can be violently agitated by rolling the rod back and forth between the thumb and index finger. The rod should remain in the tube until the proteins have been precipitated.

added. The tube is kept 1 to 1.5 hours in a water bath at 40 to 45° C. during which time the contents are frequently mixed by stirring.*

Identical results are obtained with the following slightly modified procedure: 0.5 c.c. of *N* HCl is added to the laked blood, which is followed by incubation with 0.2 to 0.25 Gm. of phosphatase for one hour. The mixture is then further acidified with 1.5 c.c. of *N* HCl, after which it is heated ten minutes in a boiling water bath with frequent stirring, and then cooled.

A blank is prepared by adding the same reagents in the order indicated. If the alternate procedure is used, the phosphatase and 1 drop of *N* HCl are added to 25 c.c. of water. The mixture is incubated at 40 to 45° C. This blank, rather than the salt blank, is necessary to correct for the small amount of thiamin present in the phosphatase.

Precipitation of Proteins.—Ten c.c. of HPO_3 solution are next added. The volume is adjusted to the 50 c.c. mark and the contents are well mixed. The mixture is centrifugated fifteen minutes. The precipitate may contain some adsorbed thiamin which may be recovered by re-extraction. It is therefore emulsified with 2.5 c.c. of HPO_3 solution and the volume is again brought to 50 c.c.

Adsorption and Elution.—The necessary adsorption columns are prepared some time before the analysis and kept in a beaker of water until ready for use. A plug of glass wool (Pyrex No. 719) is pushed to the bottom of the Hennessy tube;† the tube is filled with water, and small quantities of moist zeolite are added until an excess, forming a layer several mm. deep, is present in the bell above the column. The adsorbent is more uniformly packed when floated into place in this manner. The excess of zeolite in the bell prevents clogging of the column by solid or colloidal material. Furthermore, it prevents access of air into the column, provided the drained column is not jarred. Just before use, water is drawn from the beaker upward through the column into the bell. The column is then placed in the rack† and allowed to drain. This assures a rapidly draining column which is free from air.

Brom phenol blue indicator is now added to the combined extracts contained in a 150 c.c. extraction flask, and the reaction is adjusted to 3.0 to 3.5 by means of *N* NaOH (or M $NaHCO_3$). The extract is then passed through the column. After passage of all of the extract, the flask is rinsed with several successive portions of about 5 c.c. each of distilled water, and the washings are sent through the column. The fluid is allowed to drain completely after each of the washings. Small portions of acidified 25 per cent NaCl solution are now added until exactly 25 c.c. of eluate have been collected in a calibrated test tube such as is used in the determination of nonprotein nitrogen.

*More or less acid, and correspondingly more or less bicarbonate solution, are necessary when tissues, feces, foods, or other materials are analyzed. The volume of acid and the length of heating in the water bath are different for each class of substance. In every case, the optimum conditions for maximum yield and recovery of added thiamin must be determined. When this is done, the procedure given by us can be applied generally to all materials. In the case of substances rich in thiamin, the contents of the tube are transferred after the digestion to a 250 c.c. volumetric flask; 15 c.c. of HPO_3 solution are added, and a suitable aliquot is analyzed as described below.

**The tube of Hennessy with the long (125mm.) stem is preferred to others which are now being sold. With the coarser mixture of zeolite recommended by us, more uniform results are obtained when the longer stemmed columns are used.

†The rack consists of a 2" x 2" board which is mounted approximately 10 inches above the table. Holes ⅝" in diameter are spaced 4 inches apart.

Melnick and Field, Hennessy and Cerecedo, and others recommend that the solution be heated and then passed through the column; the elution is carried out with hot 25 per cent solution of KCl. Heat is not necessary when a rapidly draining column, prepared as described by us, is used. *The adsorption of thiamin is equally complete from hot or cold solutions, and the vitamin is recovered just as well with cold 25 per cent solution of NaCl.*

The removal of ions by zeolites is most efficient at neutral or slightly alkaline reaction.[21] At this reaction the recovery of thiamin may be smaller than at pH 3, however, especially if the solution is heated. This is due perhaps to the sensitivity of the vitamin to alkali. For this reason the neutralization with strong NaOH must be carried out carefully.

Oxidation to Thiochrome.—Two aliquots of 10 c.c. each of the eluate are transferred to Hennessy reaction vessels of 50 c.c. capacity. Five c.c. of oxidation mixture are rapidly added, with mixing of the contents. This is followed immediately with 13 c.c. of iso-butyl alcohol. The contents are then shaken ninety seconds. Four oxidations are carried out in rapid succession. After centrifugation for a few minutes at low speed, the lower layer is drawn off, and 3 to 4 small scoopfuls (about 0.2 Gm. each) of anhydrous sodium sulfate are added. *The solvent must not be shaken with the salt, as this may produce an emulsion.* When added in this manner, the first scoopful of sulfate entraps the remaining solution at the bottom of the vessel. This is covered by successive layers of less hydrated sulfate which can then remove the water more effectively from the alcoholic phase. The extract becomes crystal-clear on standing one-half minute or so. If not clear, the mixture is very gently rotated, and 1 or 2 scoopfuls of the sulfate are added and the mixture is again centrifuged for one minute. Without further waiting, the fluorescence is then determined.

The blank is oxidized and extracted in a similar manner.

The thiamin content is obtained from a calibration curve representing the increase of fluorescence after the addition of 0.2, 0.4, 0.6, 0.8, or 1.0 microgram of thiamin to blood samples which have been analyzed by the procedure.

It will be noted that the oxidation is carried out at a considerably higher alkalinity than that used by previous workers, although a trend toward the use of larger quantities of alkali is noted in recent papers.[19, 22] More consistent results are obtained at this high alkalinity, due perhaps to the greater ionic concentration which favors the extraction of thiochrome by iso-butyl alcohol.

EXPERIMENTAL

Results of analysis of blood taken from 7 women and 29 men, a total of 36 subjects, are shown in Table I. The samples were collected from 10 to 12 in the morning and from 2 to 5 o'clock in the afternoon. They were thus taken from two to five hours after a meal. None of the samples were taken under basal conditions. These conditions were chosen because they were most convenient to the majority of subjects. They represent also the conditions under which blood is most conveniently taken from large groups of subjects in a nutritional study. In the order of numbers, the subjects consisted of research workers, students, internes, physicians, and janitors. None had received any vitamin preparations within the preceding month. All were in apparent good health, and all were receiving adequate diets. The blood of the women contained from

3.0 to 9.2, and that of the men from 3.8 to 11.2 micrograms per 100 c.c. The arithmetic mean of the results from the women was 5.60, and from the men, 5.73 micrograms per 100 c.c. The mean of all of the determinations was 5.70 micrograms per 100 c.c.

TABLE I

THIAMIN CONTENT OF WHOLE BLOOD OF HUMAN SUBJECTS

	WOMEN	MEN
Subjects	7	29
Determinations	18	57
Range, micrograms per 100 c.c.	3.0 to 9.2	3.8 to 11.2
Arithmetic mean, micrograms per 100 c.c.	5.60	5.73

Diet can affect the thiamin content of the blood. The level is fairly stable on a constant diet. Thus, the blood of subject B. J. contained 6.1 micrograms per 100 c.c. at the beginning of an experiment in which the subject received a high caloric diet which contained from 1500 to 1900 micrograms of thiamin per day. The thiamin levels, determined every other day during the second and third weeks of the experiment, were 4.5, 4.3, 4.5, 5.5, 5.0, 4.5, and 4.3 micrograms per 100 c.c. On a diet containing approximately 2500 micrograms of thiamin per day, the blood of subject T. F. taken at monthly intervals contained 7.0, 9.9, and 9.9 micrograms of thiamin per 100 c.c. After two weeks, however, during which the subject received a diet containing about 300 micrograms of thiamin per day, the blood level fell to 2.8 micrograms per 100 c.c.

The following data are representative of the results which may be obtained from apparently healthy human subjects. Samples were obtained at approximately monthly intervals. The results are expressed in micrograms per 100 c.c.: K., 5.8, 6.0, and 4.9; J. H., 5.0, 5.8, and 3.9; E. A., 5.1, 8.0, and 5.1; E. G., 4.4, 7.5, 6.1, and 6.8; H., 9.2, 5.4, 3.0, and 4.1.

SUMMARY AND CONCLUSIONS

A procedure is described for the determination of thiamin in 5 c.c. of oxalated whole blood by the thiochrome method.

The blood of 36 human subjects in apparent good health and receiving an adequate diet contained from 3.0 to 11.2, with an average of 5.70 micrograms per 100 c.c. These results agree substantially with those obtained by previous workers with the cocarboxylase and fermentation methods.

REFERENCES

1. Melnick, D., Field, H., Jr., and Robinson, W. D.: A Quantitative Chemical Study of the Urinary Excretion of Thiamin by Normal Individuals, J. Nutrition 18: 593, 1939.
Robinson, W. D., Melnick, D., and Field, H., Jr.: Urinary Excretion of Thiamin in Clinical Cases and the Value of Such Analyses in the Diagnosis of Thiamin Deficiency, J. Clin. Investigation 19: 399, 1940.
2. Mason, H. L., and Williams, R. D.: The Urinary Excretion of Thiamin as an Index of the Nutritional Level: Assessment of the Value of a Test Dose, J. Clin. Investigation 21: 247, 1942.
3. Ochoa, S., and Peters, R. A.: Vitamin B_1 and Cocarboxylase in Animal Tissues, Biochem. J. 32: 1501, 1938.
4. Goodhart, R. S., and Sinclair, H. M.: The Estimation of Cocarboxylase (Vitamin B_1 Diphosphate Ester) in Blood, Biochem. J. 33: 1099, 1939.
5. Goodhart, R. S., and Sinclair, H. M.: Deficiency of Vitamin B_1 in Man as Determined by the Blood Cocarboxylase, J. Biol. Chem. 132: 11, 1940.

6. Goodhart, R. S.: A Revaluation of the Method Described by Goodhart and Sinclair for the Determination of Blood Cocarboxylase Values, J. Biol. Chem. 135: 77, 1940.
7. Schultz, A. S., Atkin, L., and Frey, C. N.: A Fermentation Test for Vitamin B_1, J. Am. Chem. Soc. 59: 948, 2457, 1937.
8. Goodhart, R. S., and Nitzberg, T.: The Thiamin Content of Human Blood and Urine as Determined by the Fermentation Method, J. Clin. Investigation 20: 625, 1941.
9. Wortis, H., Goodhart, R. S., and Bueding, E.: Cocarboxylase, Pyruvic Acid, and Bisulfite-Binding Substances in Children, Am. J. Dis. Child. 61: 226, 1941.
10. Peters, R. A.: Vitamin B_1 and Blue Fluorescent Compounds, Nature, London 135: 107, 1935.
11. Barger, G., Bergel, F., and Todd, A. R.: A Crystalline Fluorescent Dehydrogenation Product From Vitamin B_1, Nature, London 136: 259, 1935.
12. Kuhn, R., Wagner-Jauregg, T., and von Klavern, F. W.: Über einen gelben, schwefelhaltigen Farbstoff aus Hefe, Ztschr. f. physiol. Chem. 234: 196, 1935.
13. Jansen, B. C. P.: A Chemical Determination of Aneurin (= Vitamin B_1) by the Thiochrome Reaction, Rec. trav. chim. 55: 1046, 1936.
14. Kinnersley, H. W., and Peters, R. A.: Note Upon the Preparation of Crude Cocarboxylase from Vitamin B_1 by Yeast, Biochem. J. 32: 697, 1938.
15. Tauber, H.: The Interaction of Vitamin B_1 in Enzymic Reactions, J. Biol. Chem. 123: 499, 1938.
16. Hennessy, D. J., and Cerecedo, L. R.: The Determination of Free and Phosphorylated Thiamin by a Modified Thiochrome Assay, J. Am. Chem. Soc. 61: 179, 1939.
Hennessy, D. J.: Chemical Methods for the Determination of Vitamin B_1, Indust. & Engin. Chem., Anal. Ed. 13: 216, 1941.
17. Melnick, D., and Field, H., Jr.: Chemical Determination, Stability, and Form of Thiamin in Urine, J. Biol. Chem. 130: 97, 1939.
18. Ferrebee, J. W., and Carden, G. A.: A Procedure for the Routine Determination of Vitamin B_1 in Urine, J. Lab. & Clin. Med. 25: 1320, 1940.
19. Conner, R. T., and Straub, G. J.: Determination of Thiamin by the Thiochrome Reaction, Indust. & Engin. Chem. 13: 380, 1941.
20. Determination of Thiamin Hydrochloride by the Thiochrome Method, With the Adaptation of the Hennessy and Cerecedo Procedure, as Carried Out in the Laboratories of Merck and Company, Inc., Rahway, N. J., 1941.
21. Behrman, A. S., and Gustafson, H.: Effect of Low-pH Waters on Zeolites, Indust. & Engin. Chem. 28: 1279, 1936.
22. McFarlane, W. D., and Chapman, R. A.: An Improved Thiochrome Method for the Estimation of Vitamin B_1, Canad. J. Research B. 19: 136, 1941.

A METHOD FOR DETERMINING CEREBROSPINAL FLUID PROTEIN BY THE PHOTOELECTRIC COLORIMETER*

ANDRÉ CIPRIANI, M.D., C.M., AND DORIS BROPHY, LIC. ES SC., MONTREAL, CANADA

THE estimation of the protein content of cerebrospinal fluid has become an increasingly important part of laboratory routine. The ideal method should combine accuracy with speed of operation and the use of minimal quantities of spinal fluid. The method of Denis and Ayer, as modified by Ayer, Dailey, and Fremont-Smith,[1] has proved very satisfactory, but the accuracy of the determination depends upon the ability of the technician to match turbidities in the ordinary colorimeter.

The photoelectric colorimeter has eliminated this personal factor, and turbidimetric methods would seem to be ideally suited to the instrument. Looney and Walsh[2] described such a method, using 2 c.c. of spinal fluid. Ikeda and Hanson[3] presented a similar method for further trial and evaluation. The latter method requires the use of 3 c.c. of fluid. Neither of these papers mentions the error introduced by color in some pathologic fluids.

The following method is a modification of that presented by Ayer, Dailey, and Fremont-Smith[1] for the standard colorimeter. One c.c. of fluid is used instead of the original 0.6 c.c. in order to simplify pipetting.

METHOD

One c.c. of cerebrospinal fluid is accurately measured into a test tube and 1 c.c. of 5 per cent sulphosalicylic acid is added. The tube is inverted or rotated gently to ensure thorough mixing and is allowed to stand at least five minutes before reading. The tube is then inverted again and the mixture is transferred to a small colorimeter tube after the latter has been rinsed with 2 to 3 drops of the mixture. The tube is placed in the colorimeter and the deflection is read on the galvanometer.

The center setting is determined with a tube containing 1 c.c. of distilled water and 1 c.c. of 5 per cent sulphosalicylic acid and can be used for all fluids that are clear and colorless. We have found a preliminary "warming up" period of five minutes to be sufficient for the colorimeter, provided care is taken to adjust the center setting before each reading.

All fluids which are not clear and colorless have been found to give false high results due to the added absorption of light by color. This can be compensated for by adding 1 c.c. of water to 1 c.c. of the fluid (in whatever dilution has been used for precipitation) and using this as a blank to determine the center setting. Preliminary detection of color depends upon the eye of the technician, but where there is the slightest doubt a blank reading should be made in this way. Such readings may show a deflection of as much as ten divisions

*From the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal.

Received for publication, February 4, 1942.

on the galvanometer above the center setting (approximately 65 on a galvanometer scale divided in 100 equal parts) obtained with the water and acid blank. In contrast to this, a series of cerebrospinal fluid plus water blanks was run on 15 "clear and colorless" fluids. Fourteen of these varied only one-half of a division, and the fifteenth, three-fourths of a division, from the deflection of the water plus acid blank.

Protein concentrations from 12 to 110 mg. per 100 c.c. (17 to 76 per cent light absorption) can be estimated directly. Fluids containing higher concentrations must be diluted so that the reading falls within those limits.

APPARATUS

The apparatus consists of a miniature colorimeter of the Evelyn type[4] built to accommodate small Pyrex test tubes (8 × 75 mm.). These tubes were carefully tested for uniformity and only those conforming to the standard set by the Rubicon Company were used.

The miniature attachment for the photoelectric colorimeter is constructed as follows: Light from a 6.3 volt lamp passes through a built-in filter (Rubicon 520) and then through the solution by way of a slit 0.25 inch wide and 0.75 inch high. The test tube is so aligned that the bottom of the slit is just above the rounded portion of the tube. A removable, lightproof cap is necessary to prevent stray light from reaching the photocell via the top of the tube. The light intensity is recorded by means of a General Electric blocking layer photocell.

The instrument is included in the case of the standard Evelyn colorimeter and makes use of the same light control circuit and galvanometer. A suitable switch allows the convenient use of either instrument.

CALIBRATION

A standard protein solution was prepared according to the method of Ayer, Dailey, and Fremont-Smith.[1] Dilutions were made to give eleven standard solutions ranging in concentration from 12 to 110 mg. per 100 c.c. The protein content of each was determined in duplicate by the micro-Kjeldahl method and also the light absorption of each, after precipitation with sulphosalicylic acid, was measured in the photoelectric colorimeter. Using the actual readings on the galvanometer scale as ordinate and the concentration of protein (as determined by micro-Kjeldahl) as abscissa, points were established on graph paper and a curve drawn. This curve has been used to estimate the protein in all fluids of unknown concentration.

Duplicate determinations of protein were made on numerous samples of cerebrospinal fluid both by the photoelectric method and by micro-Kjeldahl. As noted by Ayer, Dailey, and Fremont-Smith,[1] difficulty was encountered in obtaining satisfactory checks with the micro-Kjeldahl when the nonprotein nitrogen was subtracted from the total nitrogen and the result multiplied by the factor 6.25. More consistent results were obtained by precipitating the protein with trichloracetic acid and determining the nitrogen content of the precipitate directly. This method has been compared with the direct gravimetric method by Guillaumin et al.[5] and has been found to check closely with it. It has the added advantage of requiring only two micro-Kjeldahl determinations instead of four for duplicate estimations.

TABLE I

TOTAL PROTEIN, MG. PER 100 C.C.

PHOTOELECTRIC	MICRO-KJELDAHL (TOTAL N. MINUS N.P.N.)
14.3 & 14.5	16.9 & 14.4
15.6 & 15.8	21.9 & 22.5
20.2 & 20.2	21.2 & 24.4
20.5 & 20.8	18.1 & 19.3
22.3 & 21.8	31.3 & 30.0
27.5 & 27.5	31.3 & 32.5
39.5 & 39.5	43.1 & 40.6
48.0 & 48.7	50.6 & 46.9
49.2 & 48.7	51.3 & 45.0
55.0 & 55.3	54.4 & 53.1
57.0 & 57.3*	63.8 & 64.4
57.8 & 57.0	60.6 & 55.6
63.2 & 64.2	58.1 & 63.1
72.8 & 73.5	83.1 & 88.1
73.5 & 74.2	78.1 & 74.4
82.0 & 81.3	79.2 & 77.9
89.0 & 87.5	92.5 & 87.5
90.3 & 91.3	101.9 & 103.1

*Xanthochromic fluid—center setting adjusted.

TABLE II

TOTAL PROTEIN, MG. PER 100 C.C.

PHOTOELECTRIC	MICRO-KJELDAHL (PROTEIN NITROGEN)
12.5 & 12.3	12.5 & 9.8
14.7 & 14.9	17.5 & 17.9
19.5 & 19.7	24.1 & 20.1
21.3 & 21.1	20.1 & 20.7
27.8 & 27.8	28.4 & 25.2
28.7 & 28.5	31.5 & 32.6
39.4 & 39.2	36.6 & 37.0
44.3 & 44.0	45.5 & 45.5
50.7 & 50.7	47.7 & 47.3
54.2 *	56.4 & 56.0
59.3 & 59.7*	55.6 & 57.8
65.3 & 65.7*	60.4 & 62.1
68.2 & 69.2	63.8 & 69.1
98.0 & 99.6*	93.6 & 91.0
189.0 & 192.4	184.6 & 181.1
422.5 & 425.5*	428.7 & 446.3
6,480 & 6,430†	6,191 & 5,906

*Xanthochromic fluids—center setting adjusted.
†Cyst fluid—center setting adjusted.

RESULTS

All the figures obtained in the course of the experiment are presented in Tables I and II. It will be noted that the results of photoelectric determinations are in more close agreement with the micro-Kjeldahl results in Table II (protein nitrogen) than is the case in Table I (total nitrogen minus nonprotein nitrogen). Duplicate figures in the photoelectric method are remarkably consistent, and where the figures by micro-Kjeldahl determination were not in such close agreement the photoelectric results will be found to check fairly closely with one or the other of them. In only two instances (the second and the fourteenth) in Table II were the duplicate values for each method in close agreement while showing a distinct difference between the two methods. In one of these the

figures by photoelectric method were higher, and in the other they were lower than the Micro-Kjeldahl results.

The method is convenient and inexpensive. A single determination requires about ten minutes and even less time per determination if a number of samples are to be tested.

SUMMARY

1. A simple method is presented for the determination of cerebrospinal fluid protein with the photoelectric colorimeter.

2. A miniature attachment for the Evelyn photoelectric colorimeter is described which will accommodate small Pyrex test tubes. This permits the determination to be carried out on 1 c.c. of spinal fluid.

3. False high results may be obtained with colored specimens of spinal fluid. A method to rule out this source of error is described.

REFERENCES

1. Ayer, J. B., Dailey, M. E., and Fremont-Smith, F.: Denis-Ayer Method for the Quantitative Estimation of Protein in the Cerebrospinal Fluid, Arch. Neurol. & Psychiat. 26: 1083, 1931.
2. Looney, J. M., and Walsh, A. I.: The Determination of Spinal Fluid Protein With the Photoelectric Colorimeter, J. Biol. Chem. 127: 117, 1939.
3. Ikeda, K., and Hanson, A.: Photelometric (Sheard-Sanford) Determinations of Blood Cyanate, Blood Sulfapyridine, Urine Sugar and Total Proteins in Spinal Fluid, Am. J. Clin. Path. Tech. Supp. 3: 192, 1939.
4. Evelyn, K. A.: Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. 115: 63, 1936.
5. Guillaumin, Ch. O., Wahl, R., and Laurencin, M. L.: Sur le dosage des albumines sériques. Comparaison de quelques résultats obtenus par pesée, par azotométrie et par réfractométrie, Bull. Soc. chim. biol. 11: 387, 1929.

DETERMINATION OF MANDELIC ACID IN URINE*

Precipitation as Cadmium Mandelate

J. H. Phillips, A.B., V. H. Wallingford, Ph.D., and A. Q. Butler, Ph.D.
St. Louis, Mo.

MANDELIC acid and salts of mandelic acid are employed in the treatment of urinary infections and after their administration the physician may find it desirable to follow the concentration of mandelic acid in the urine of the patient. In a clinical investigation[2] on the injection of solutions of sodium mandelate this laboratory was requested to supply the analytical data. The available analytical procedures for the determination of mandelic acid in urine were studied and found to be unreliable. A new gravimetric method was developed in which the mandelic acid in the urine is extracted with ether, converted to the sodium salt for extraction and concentration in an aqueous solution, and precipitated and weighed as cadmium mandelate. The method may be carried out

*From the Mallinckrodt Chemical Works, St. Louis.
Received for publication, March 1, 1943.

fairly rapidly on a semimicro scale with 10 c.c. samples of urine, or it may be applied as a macro procedure with 100 c.c. samples. The concentration range of 0.1 to 2.0 Gm. of mandelic acid in 100 c.c. of sample was studied since this represents the normal range of concentrations encountered during mandelic acid therapy.

In 1909 Neubauer[5] determined the mandelic acid excreted by dogs after feeding experiments. The acid was extracted from the urine by ether and, after purification, was titrated with 0.1N alkali. Rosenheim,[11] who introduced mandelic acid therapy in 1935, determined mandelic acid in the urine of his patients by extracting with ether after acidifying the specimen with phosphoric acid and saturating with ammonium sulfate. The residue obtained by evaporating the ether was weighed as mandelic acid. McMahon[6] combined the Rosenheim extraction method with the titration method used by Neubauer. Montenbruck,[7] and also Garry and Smith,[3] determined the extracted *d* or *l* mandelic acid polarimetrically. This method is not applicable in human cases, however, since the inactive *dl* mandelic acid is usually administered, and it has been shown[7] that both forms of mandelic acid are excreted simultaneously. For the assay of medicinal preparations, Stevens and Hughes,[12] and also Rosen,[10] employed continuous extraction with ether followed by titration with standard alkali. Underwood[13] recommended extraction with a mixture of two parts chloroform and one part ether, followed by titration with standard alkali.

The published methods are subject to several errors. Reimers[9] indicated that care must be taken in evaporating the ether from the extract in order to avoid loss of mandelic acid by volatilization. McMahon reported the loss of an average of 6 per cent of mandelic acid in recovery experiments from water with samples ranging from 0.5 to 4.5 Gm., and 16 per cent loss in recovery from urine with samples ranging from 0.2 to 0.3 Gm. The loss was attributed to combination with other constituents of the urine. Berger,[1] using McMahon's method, reported a loss of about 10 per cent from 0.1 gram samples in recovery of mandelic acid from urine "through the combination with other urinary constituents or through inaccuracy of the method." He also reported a loss of 20 per cent in the recovery of mandelic acid added to breast milk.

DEVELOPMENT OF GRAVIMETRIC METHOD

A study of the methods reviewed above indicated a need for a more satisfactory procedure for the determination of mandelic acid in urine. In attempting to apply Rosenheim's method of extraction from 100 c.c. portions of urine, intractable emulsions were frequently encountered, but the use of the continuous method of extraction with ether recommended by Stevens and Hughes entirely overcame this difficulty. It was found necessary to acidify the urine rather strongly to insure the complete liberation of the mandelic acid. High acidity has been shown[4] to decrease the solubility of mandelic acid in water and would be expected to facilitate its extraction by ether. The extracted mandelic acid, however, was always found to be impure, and direct titration generally gave results which were high. It was found that pure mandelic acid can be nearly quantitatively precipitated by adding concentrated hydrochloric acid or

by saturating the solution with sodium chloride, but mandelic acid recovered from urine under these conditions crystallized only slowly and incompletely, presumably on account of the inhibiting action of the accompanying impurities.

Mandelic acid extracted from urine by means of ether was found to be promptly and quantitatively precipitated as the cadmium salt from aqueous solution, furnishing the basis for a satisfactory gravimetric method. McKenzie[5] prepared cadmium mandelate and listed its solubility as 0.1 Gm. in 100 c.c. of water at 16° C. Since McKenzie did not publish his analytical data, the present authors have determined the composition of cadmium mandelate precipitated under various conditions and have redetermined its solubility.

It was noted that the physical characteristics of the crystalline cadmium mandelate formed at room temperature changed appreciably when the solution with precipitate was heated on the steam bath. The cadmium mandelate was formed first as a dense crystalline precipitate which upon heating changed to a flocculent form. Pure cadmium mandelate is white, but when precipitated from urine it may be slightly colored. It was subsequently established that both forms of precipitate had the same composition after they were dried. Samples representing each of the two forms of precipitate were prepared and analyzed. Cadmium mandelate was precipitated by adding an excess of cadmium chloride solution (50 Gm. cadmium chloride in 100 c.c. water) to a slightly acidified aqueous solution of sodium mandelate, the conditions adopted for the analytical procedure described later. In one case the cadmium mandelate was precipitated at room temperature, after which it was heated on the steam bath until the physical change in the crystalline structure was noted. The precipitate was collected on a sintered glass funnel and washed with small portions of distilled water until free from chloride (Sample No. 1). A second sample of cadmium mandelate was prepared by precipitation from a previously cooled solution of sodium mandelate, and the solution and crystals were kept in an ice bath until the washing of the precipitate was completed (Sample No. 2). The two samples were dried in a vacuum desiccator and analyzed by simultaneous microcombustion for cadmium, carbon, and hydrogen. Cadmium was converted to the oxide during the course of the carbon and hydrogen combustion, and the percentage of cadmium was calculated from the residue of cadmium oxide remaining in the platinum boat. A complete combustion of the cadmium mandelate was easily obtained at a temperature well below the decomposition temperature of cadmium oxide, 900 to 1000° C. During the combustion the boat was not heated above a dull red heat. The results are shown in Table I. The values obtained for percentage composition indicated that $Cd(C_8H_7O_3)_2$ is the correct formula for both forms of precipitate. It is probable that the differences in physical characteristics are associated with hydrate formation.

Solubility determinations were made using portions of the purified cadmium mandelate samples described above, at 25° C., in water and in dilute acetic acid (the conditions recommended in the analytical procedure). Approximately 0.2 Gm. of cadmium mandelate was placed in a 200 c.c. Kjeldahl flask together with 100 c.c. of distilled water. The flask was fitted with a motor-driven glass stirrer and clamped in a constant temperature water bath. The contents of the flask were stirred slowly for three hours, at which time a sample of 15 c.c. was with-

drawn through a sintered glass filter stick into a calibrated pipette and transferred to a previously tared micro-evaporating dish. The water was cautiously evaporated on a steam bath, the dish and residue dried in a vacuum desiccator over phosphorus pentoxide and again weighed. The increase in weight was calculated to grams of cadmium mandelate per 100 c.c. of solution. A second portion was withdrawn three hours later to determine whether the equilibrium point had been attained. As a check on the results, a determination was made in which the original mixture was heated to 100° C. and then cooled to 25° C. Another determination was made in an acetic acid solution pH 4.11. The results of these determinations are shown in Table II. The average value for solubility, 0.08 Gm. in 100 c.c. of solution, is slightly lower than the value reported by McKenzie. The solubility was found to be only slightly greater in acetic acid than in water.

TABLE I

ANALYSIS OF CADMIUM MANDELATE

	Cd	C	H
	PER CENT	PER CENT	PER CENT
Theory $Cd(C_8H_7O_3)_2$	27.1	46.3	3.4
Sample No. 1	27.2	(47.5)	3.7
	27.3	46.3	3.7
	27.3	46.6	3.7
Sample No. 2	27.2	46.7	3.2
	27.1	46.3	(4.0)
	26.9	46.0	3.6

TABLE II

SOLUBILITY OF CADMIUM MANDELATE AT 25° C.

	PURIFIED CADMIUM MANDELATE SAMPLE USED FOR TEST	SOLUBILITY IN GRAMS PER 100 C.C. OF SOLUTION
Solution in distilled water adjusted to 25° C. from room temperature	No. 1	0.079
	No. 2	0.079
Solution in distilled water heated to 100° C and cooled to 25° C.	No. 2	0.078
Solution in acetic acid, pH 4.11, adjusted to 25° C. from room temperature	No. 1	0.089

The most convenient method of extraction for small samples of urine was found to be repeated shaking of the sample with ether in a 50 c.c. separatory funnel. Several solvents and mixtures of solvents, such as ether, chloroform, normal butyl alcohol, normal amyl alcohol, benzene, and carbon tetrachloride, were tried, but alcohol-free ether was found to be the best. When extracting with ether it was found necessary to avoid the use of grease on the stopcock of the separatory funnel, but the stopcock may be moistened with distilled water before the sample is introduced. Two satisfactory procedures were developed for transferring the extracted mandelic acid to a water solution preparatory to precipitation as the cadmium salt. In the semimicro procedure, the ether extract was neutralized with sodium hydroxide and the ether evaporated from the mixture. In the macro procedure, it was found expedient to extract the ether

solution with dilute sodium hydroxide and expel dissolved ether from the sodium hydroxide fraction by warming on the steam bath. By converting the mandelic acid to the sodium salt before evaporating the ether in both procedures all possibility of loss of the acid by volatilization was eliminated.

In order to avoid extracting unnecessary amounts of impurities from the urine samples by repeated shaking in the 50 c.c. separatory funnel, a series of extractions was made on 10 c.c. aliquot portions of a solution containing a known amount of mandelic acid. The results showing per cent recovery in these experiments are presented in Table III. It was found that five extractions are adequate.

TABLE III

ETHER EXTRACTION OF MANDELIC ACID FROM WATER
(0.2000 GM. MANDELIC ACID DISSOLVED IN 10 C.C. WATER)

NUMBER OF ETHER EXTRACTION	WEIGHT MANDELIC ACID RECOVERED GRAMS	RECOVERY PER CENT
3 × 30 c.c.	0.1768	88.4
5 × 30 c.c.	0.1989	99.5
7 × 30 c.c.	0.1998	99.9

METHOD OF ANALYSIS BY THE SEMIMICRO PROCEDURE

Reagents.—All chemicals used are Mallinckrodt reagents of the grade specified: Sulfuric acid A.R., diluted 1:1 by volume with distilled water; cadmium chloride solution, 50 Gm. cadmium chloride anhydrous A.R. in 100 c.c. water; sodium hydroxide 1N; chlorphenol red indicator, 0.1 per cent in 95 per cent ethyl alcohol; acetic acid glacial A.R.; ether anhydrous A.R.

Procedure.—Measure a 10 c.c. portion of the urine specimen into a 50 c.c. separatory funnel, add 1 c.c. of sulfuric acid (1:1), 30 c.c. anhydrous ether, and shake vigorously for three minutes. Allow the two layers to separate and withdraw the lower aqueous layer into a second 50 c.c. separatory funnel. Use necessary precautions to transfer all of the aqueous layer and ether-water suspension that does not separate readily. Wash the final drops from the stem into the second funnel with a stream of ether from a wash bottle. Transfer the ether layer remaining in the funnel into a 50 c.c. beaker; add 3 c.c. water, 5 drops chlorphenol red indicator, and a slight excess of 1N sodium hydroxide; and place on the steam bath with a current of air directed into the beaker. Allow all of the ether to evaporate thus leaving the mandelic acid in the aqueous layer as the sodium salt. While this first ether layer is evaporating, extract the aqueous solution in the second separatory funnel with another 30 c.c. of anhydrous ether and transfer this extract to the beaker in which the first extract was evaporated. Repeat the extraction and evaporation of the ether layer until five extractions have been made. The aqueous solution of mandelic acid in the beaker must be kept alkaline during these operations by the addition of sodium hydroxide, 0.5 to 1 c.c. at a time, as indicated by the chlorphenol red. Evaporate the aqueous solution of sodium mandelate thus obtained to about 3 c.c. and transfer quantitatively to a 10 c.c. micro beaker which has been previously weighed with a sintered Pyrex or porcelain filter stick.

Again evaporate the combined solution and washings on the steam bath to a volume of about 3 c.c., adjust the pH to approximately 5 by the addition of glacial acetic acid dropwise until the yellow color of the chlorphenol red indicator starts to appear, and finally add 0.4 c.c. of cadmium chloride solution. Evaporate the contents of the beaker to dryness on the steam bath, take up the residue in 3 c.c. of water, and allow the beaker and contents to stand for about thirty minutes with occasional agitation to obtain solution of the water-soluble salts. Filter the solution through the previously weighed filter stick. Rinse the inside of the beaker and filter stick with two 0.5 to 1.0 c.c. portions of water with successive withdrawal of each through the filter stick to remove all traces of soluble cadmium and sodium salts. Dry the beaker and filter stick in a vacuum desiccator over phosphorus pentoxide and weigh. Multiply the weight of cadmium mandelate by 0.7338 to convert to mandelic acid.

METHOD OF ANALYSIS BY THE MACRO PROCEDURE

Reagents.—The reagents are the same as those listed above for the semimicro method.

Procedure.—Any of the commonly used types of apparatus for continuous extraction by an immiscible liquid should suffice for this extraction. The type used here consisted of a 500 c.c. round bottom flask for a boiler, a 125 c.c. distillation flask for holding the sample, and a reflux condenser. Connections were made so that the condensed ether passed through a tube to the bottom of the flask holding the sample, overflowing through the side arm back to the boiling flask. The tube which returned the ether extended below the surface of the liquid in the boiler flask.

Introduce a 100 c.c. portion of the urine specimen and 10 c.c. sulfuric acid (1:1) into the distillation flask. Introduce 300 c.c. of anhydrous ether into the round-bottom flask. Connect the refluxing tube and the condenser to the two flasks, and connect the side arm of the distillation flask to the return tube so that excess ether will drain back into the round-bottom flask. Place the round-bottom flask on a steam bath or electric hot plate and allow the extraction to proceed for twelve hours, or overnight if convenient. At the end of the refluxing period, remove the round-bottom flask from the extraction setup, add 5 or 6 drops of chlorphenol red indicator and sufficient 1N sodium hydroxide to maintain the pink end-point of the indicator. Stopper the flask with a rubber stopper and shake vigorously for two or three minutes to insure that the end point has been reached. Place the round-bottom flask and contents on a steam bath and allow about one-half of the ether to evaporate. Transfer the ether-water mixture quantitatively to a 250 c.c. separatory funnel and add sufficient water to bring the total volume of the aqueous layer to 15 c.c. Shake the mixture vigorously for three minutes. Add more alkali, if necessary, to keep the aqueous layer alkaline. Withdraw the aqueous layer into a 250 c.c. beaker and rinse any adhering drops on the tip of the separatory funnel into the beaker with a stream of ether from the wash bottle. Repeat the extraction of the ether layer in the separatory funnel three more times, using 1 c.c. of 1N sodium hydroxide and 4 c.c. of water. Transfer each water extract to the same beaker. Place the

beaker on a steam bath and allow the ether to evaporate completely from the aqueous layer.

Adjust the pH of the water-extract to approximately 5 or 6 by the addition of sufficient glacial acetic acid to just reach the yellow end point of the chlorphenol red indicator. Precipitate the mandelic acid by adding 4 c.c. of cadmium chloride solution. Place the beaker containing the precipitate on a steam bath and allow the solution to evaporate to dryness. Add 10 c.c. of water to the residue, mix thoroughly, and allow to stand at room temperature for thirty minutes with occasional stirring. Filter the solution into a weighed sintered glass funnel, transferring the precipitate quantitatively into the funnel. Wash the precipitate with three 1 to 2 c.c. portions of water. Dry the funnel in a vacuum desiccator over phosphorus pentoxide and weigh. Multiply the weight of cadmium mandelate by 0.7338 to convert to mandelic acid.

DISCUSSION

The mandelic acid content of urine may be determined by either of the two procedures described above. In Tables IV and V are shown the results of experiments in which known quantities of mandelic acid were added to water and urine respectively and then recovered by the semimicro procedure. In Table VI are shown the results of experiments in which known quantities of mandelic acid were added to urine and recovered by the macro procedure. Although the methods appear to give equally satisfactory results over the range of concentrations studied, the semimicro method has the advantage that it requires considerably less elapsed time per analysis. It was found that five or six samples may be completed by the semimicroprocedure by an analyst in an eight-hour day. Another distinct advantage of the semimicromethod is that it permits the use of a smaller sample.

There is always an unavoidable uncertainty connected with the extraction of urine samples due to the lack of uniformity in the composition of different samples. It is conceivable that the cadmium mandelate procedure might be influenced by constituents in the urine, but significant interference by constituents of either normal or pathologic urine seems unlikely. A number of samples of urine originating from patients before the initiation of mandelic acid therapy were analyzed by the cadmium mandelate precipitation method, and in every case the complete absence of mandelic acid and all interfering impurities was indicated. Oxalic acid forms an insoluble cadmium salt, but it exists in urine in traces only. Hippuric acid is also only a minor constituent of human urine. The cadmium salts of benzoic acid and the common aliphatic acids are sufficiently soluble to avoid interference. Beta-hydroxybutyric acid, which may be present in urine under certain conditions, failed to yield a precipitate with cadmium chloride.

The results obtained by the cadmium mandelate precipitation method are subject to a slight error due to the solubility of the cadmium mandelate, but a high degree of accuracy is not required in a urinalysis and the solubility error is probably not significant except in samples where the mandelic acid concentration is very low. If an analysis required a more accurate determination the solubility error could be corrected either by calculation or manipulation.

TABLE IV

RECOVERY OF MANDELIC ACID FROM WATER

SEMIMICRO PROCEDURE

WEIGHT OF MANDELIC ACID ADDED TO 10 C.C. WATER GRAMS	WEIGHT MANDELIC ACID RECOVERED TRIAL 1 GRAMS	TRIAL 2 GRAMS
0.0100	0.0119	0.0131
0.0500	0.0523	0.0521
0.1000	0.1037	0.1013
0.1500	0.1481	0.1514
0.2000	0.2015	0.1980

TABLE V

RECOVERY OF MANDELIC ACID FROM URINE

SEMIMICRO PROCEDURE

WEIGHT OF MANDELIC ACID ADDED TO 10 C.C. URINE GRAMS	WEIGHT OF MANDELIC ACID RECOVERED			
	URINE SAMPLE 1 GRAMS	URINE SAMPLE 2 GRAMS	URINE SAMPLE 3 GRAMS	URINE SAMPLE 4 GRAMS
0.0100	0.0105	0.0109	0.0093	0.0095
0.0500	0.0485	0.0508		
0.1000	0.1024	0.1049		
0.1500	0.1496	0.1511		
0.2000	0.2039	0.1995		

TABLE VI

RECOVERY OF MANDELIC ACID FROM URINE

MACRO PROCEDURE

WEIGHT OF MANDELIC ACID ADDED TO 100 C.C. URINE GRAMS	WEIGHT OF MANDELIC ACID RECOVERED			
	URINE SAMPLE 1* GRAMS	URINE SAMPLE 3* GRAMS	URINE SAMPLE 5 GRAMS	URINE SAMPLE 6 GRAMS
0.1000	0.1421	0.0911		
0.5000	0.5081			
1.000	1.011		0.953	0.977
1.500	1.569			
2.000	2.059			

*Same urine samples as reported in Table V.

From about one hundred and thirty analyses for mandelic acid in urine by the semimicro method performed in the course of a clinical investigation[2] only two specimens formed emulsions that could not be separated. Upon inquiring about the history of the specimens it was learned that they had originated from patients who had been given mercury therapy along with the sodium mandelate, which may have been responsible for the production of an emulsifying substance in the urine. These two specimens were easily extracted by a semimicro adaptation of the continuous extractor described in the procedure for the macro method. A 100 c.c. round-bottom flask and a 50 c.c. distilling flask were used in the setup. Recovery experiments on known quantities of mandelic acid added to normal urine established the fact that four to five hours was sufficient time for the continuous extraction of 10 c.c. samples.

In Table VII is shown a series of results on case specimens of urine in which the samples were analyzed for mandelic acid by both the semimicro and macro procedures.

The isolation and determination of mandelic acid as cadmium mandelate may be of value in the analysis of other solutions or mixtures such as elixirs or tablets.

TABLE VII

COMPARISON OF CASE SPECIMENS BY SEMIMICRO AND MACRO METHODS

URINE SPECIMEN	WEIGHT OF MANDELIC ACID FOUND PER 100 C.C. URINE	
	SEMIMICRO GRAMS	MACRO GRAMS
No. 1	0.19	0.20
No. 2	1.62	1.64
No. 3	0.35	0.32
No. 4	0.12	0.11
No. 5	0.03	0.04

SUMMARY

A method for the determination of mandelic acid in urine is presented and its use for other types of samples is suggested. The procedure is based on the extraction of the acid with ether, conversion to the sodium salt, and precipitation as cadmium mandelate. A semimicro and a macro procedure are given and two methods of extraction are described. The methods are accurate and require no special equipment or reagents. Several determinations may be carried out simultaneously.

The composition of cadmium mandelate has been confirmed by analysis and its solubility redetermined. A method is reported for the determination of cadmium as the oxide simultaneously with the determination of carbon and hydrogen by combustion.

The authors wish to express their thanks to Drs. Grayson Carroll and Reese Coleman for the privilege of collaborating with them on this investigation and to Allen G. King, librarian at the Mallinckrodt Chemical Works, for his assistance in searching the literature.

REFERENCES

1. Berger, H.: Excretion of Mandelic Acid in Breast Milk, Am. J. Dis. Child. 61: 256, 1941.
2. Carroll, Grayson, and Coleman, Reese: Paper in preparation.
3. Garry, R. C., and Smith, I. A.: The Urinary Excretion of Mandelic Acid Administered Intravenously to Cats, Biochem. J. 34: 490, 1940.
4. Knox, J., and Richards, M. S.: The Basic Properties of Oxygen in Organic Acids and Phenols; and the Quadrivalency of Oxygen, J. Chem. Soc. 115: 508, 1919.
5. McKenzie, A.: A Contribution to the Chemistry of the Mandelic Acids, J. Chem. Soc. 75: 964, 1899.
6. McMahon, S.: Intravenous Use of Mandelic Acid for Urinary Antisepsis. I. An Experimental Study of Excretion and Toxicity, J. Urol. 41: 611, 1939.
7. Montenbruck, D.: Degradation of *alpha-Amino* Acids. Behavior of Mandelic Acid in the Human Organism, Arch. f. exper. Path. u. Pharmakol. 195: 164, 1940.
8. Neubauer, O.: Decomposition of Amino Acids in Well and Diseased Organisms, Deutsches Arch. f. klin. Med. 95: 211, 1909.
9. Reimers, F.: The Determination of Novatropine and of Mandelic Acid, Dansk Tids. Farm. 12: 25, 1938.
10. Rosen, K. B.: Syrup of Ammonium Mandelate, Formula and Method of Assay, Bull. Nat. Formulary Comm. 8: 381, 1940.
11. Rosenheim, M. L.: Mandelic Acid in the Treatment of Urinary Infections, Lancet 1: 1032, 1935.
12. Stevens, A. N., and Hughes, E. J.: The Determination of Mandelic Acid in Calcium Mandelate, Monoethanolamine Mandelate and Elixir of Mandelic Acid, J. Am. Pharm. A. 28: 222, 1939.
13. Underwood, H. G.: Report on (the Determination of) Mandelic Acid, J. Assoc. Official Agr. Chem. 22: 757, 1939; see also Methods of Analysis of A. O. A. C., ed. 5, 1940, p. 610.

A RAPID AND SIMPLE TECHNIQUE FOR THE DETERMINATION OF GELATIN*

MARTHA JANOTA, M.S., CHICAGO, ILL.

THIS report deals with a technique for the determination of gelatin protein in a mixture of blood and gelatin or urine and gelatin in vivo as well as in vitro. The technique described here is merely the application of well-known methods and principles of chemistry in the separation of plasma and gelatin or urine and gelatin proteins. The present paper is reported because of the increased interest in gelatin as a blood substitute and because of the absence in the literature of suitable simple methods for the determination of gelatin.[1]

The basis for the separation of gelatin from plasma proteins is the property of gelatin protein in 95 per cent ethyl alcohol and in 5 per cent trichloracetic acid. (Gelatin throughout this paper refers to an autoclaved solution.) The addition of 95 per cent alcohol to a gelatin solution results in the protein of the gelatin being precipitated, the nonprotein constituents remaining in the supernatant fluid after centrifugation. The addition of 5 per cent trichloracetic acid to a gelatin solution does not precipitate the gelatin proteins. It is a well-known fact that 5 per cent trichloracetic acid or 95 per cent alcohol precipitates the proteins of serum or plasma, the nonprotein constituents remaining in solution.[2] Thus in a mixture of gelatin and plasma, the gelatin may be separated from the plasma by the precipitation of plasma proteins with trichloracetic acid, thereby leaving the nonprotein constituents of both plasma and gelatin and the gelatin protein in the supernatant fluid. Every sample of gelatin had a nonprotein nitrogen content varying between 30 and 75 mg. per cent after autoclaving. Prolonged autoclaving increased the nonprotein nitrogen and decreased the viscosity of the solution. A standardized procedure of autoclaving was used therefore. Now, if a total nitrogen determination is made by the micro-Kjeldahl method on the 5 per cent trichloracetic supernatant fluid and the nonprotein nitrogen is determined by the usual nesslerization method on an alcoholic filtrate of a plasma-gelatin mixture, the protein nitrogen of gelatin may be obtained by subtracting the nonprotein nitrogen from the total nitrogen. The protein nitrogen multiplied by the protein factor for gelatin of 5.25 yields the protein gelatin in the mixture.

The plasma protein of the mixture may be obtained by analyzing the gelatin-plasma mixture for total nitrogen, and then subtracting the total nitrogen of the 5 per cent trichloracetic acid supernatant which contains the gelatin protein nitrogen and the nonprotein nitrogen constituents of both gelatin and plasma. This figure represents total plasma protein nitrogen, which multiplied by the usual protein factor of 6.25 yields the protein of the plasma. It is obvious that

*From the Samuel Deutsch Serum Center of Michael Reese Hospital, Chicago.

Supported by a grant from the Edible Gelatin Manufacturers Research Society and by the Michael Reese Research Foundation.

Received for publication, March 24, 1942.

by adding the plasma protein to the gelatin protein the true combined total protein of the gelatin-plasma mixture is obtained.

These same principles may be applied to gelatin-urine analysis. In passing, it should be pointed out that the qualitative tests for protein in urine, such as 5 per cent acetic acid and sulphosalicylic acid, are not suitable for the detection of gelatin, since gelatin is not precipitated by either of these reagents.

EXPERIMENTAL

Ossein or pigskin gelatins prepared in 0.85 per cent NaCl solutions, autoclaved for one-half hour to one hour at 256° F., were used in vitro and in vivo experiments. In vitro experiments were performed by mixing gelatin and plasma in varying amounts. Total proteins were determined on the gelatin and plasma before the mixtures were made; the mixtures were analyzed for gelatin and plasma proteins, and the theoretical values were calculated. In Table I are represented the theoretical and recovered gelatin and plasma protein values.

Total nitrogen determinations were made by diluting 0.5 c.c. of the plasma-gelatin mixture to 25 c.c. with 0.9 per cent NaCl and analyzing 1 c.c. aliquots for total nitrogen by the micro-Kjeldahl method of Ma.[3]

Gelatin nitrogen was determined by precipitating the plasma proteins contained in 0.5 c.c. of the gelatin-plasma mixture with 7 c.c. of 5 per cent trichloracetic acid and by analyzing 1 c.c. portions of the supernatant for total nitrogen (G.N.) by the micro-Kjeldahl method.

Nonprotein nitrogen (N.P.N.) was determined by precipitating the gelatin and plasma proteins in 0.5 c.c. of the mixture with 7 c.c. of 95 per cent alcohol, determining the amount of N.P.N. in 5 c.c. of the supernatant by the usual methods using direct nesslerization.[4]

Protein values were obtained by the following formulas:

(T.N. – G.N.) $\times$ 6.25 = Plasma protein in grams per 100 c.c.

(G.N. – N.P.N.) $\times$ 5.25 = Gelatin protein in grams per 100 c.c.

Gelatin protein plus plasma protein = Combined protein of mixture.

In vivo experiments were performed on dogs infused with known quantities of gelatin. Gelatin and plasma protein analyses on the blood of these animals one hour, twenty-four hours, forty-eight hours, seventy-two hours, and five days later indicated that gelatin left the circulation very rapidly.[5] The analyses were made in duplicate and the values obtained agreed within ± 3 per cent error.

In order to rule out the rapid loss of gelatin from the circulation due to diffusion of gelatin into the red cells, heparinized blood was mixed with equal amounts of gelatin solution. The mixture was gently shaken for one hour, and then centrifuged, with the plasma analyzed for gelatin and plasma protein. In one experiment the red blood cells of the mixtures were repeatedly washed with 0.85 per cent NaCl; the washed cells were then frozen rapidly in carbon dioxide snow and alcohol, thawed rapidly, and analyzed for gelatin. Table II represents the results of this experiment. The theoretical values were calculated from hematocrit determinations of the blood before adding the gelatin.

DISCUSSION

From the data presented in Tables I and II, it would appear that the technique for the determination of gelatin and plasma protein in a mixture of gelatin

TABLE I

RECOVERY OF GELATIN FROM A MIXTURE OF PLASMA AND GELATIN

PROPORTION GELATIN: PLASMA	PER CENT PROTEIN CONTENTS		PER CENT GELATIN PROTEIN			PER CENT PLASMA PROTEIN			PER CENT COMBINED PROTEIN			NPN		PER CENT OF GELATIN RECOVERED
	GELATIN	PLASMA	CALCUL.	RECOVER.	DIFFER.	CALCUL.	RECOVER.	DIFFER.	CALCUL.	RECOVER.	DIFFER.	CALCUL.	RECOVER.	
1:1	6.61	4.68	3.32	3.26	-.06	2.34	2.47	+.13	5.06	5.73	+.07	64	62	98.2
1:2			2.23	1.98	-.25	3.12	3.16	+.04	5.35	5.14	-.21	50	52	88.8
1:4			1.34	1.21	-.13	3.74	3.96	+.22	5.08	5.17	+.09	38	32	90.3
1:9			0.67	0.63	-.04	4.21	4.02	-.19	4.88	4.65	-.23	30	27	94.0
2:1			4.45	4.39	-.06	1.56	1.70	+.14	6.01	6.09	+.08	78	75	98.6
1:1	4.80	5.91	2.40	2.21	-.19	2.97	3.21	+.24	5.37	5.42	+.05	79	80	92.0
1:2			1.60	1.46	-.14	3.96	4.20	+.24	5.56	5.66	+.10	81	80	91.2
1:4			0.96	0.89	-.07	4.75	4.79	+.04	5.71	5.68	-.03	83	86	92.5
3:2			2.87	2.65	-.22	2.38	2.67	+.29	5.25	5.32	+.07	78	76	92.3
1:1	6.88	6.56	3.44	3.21	-.23	3.28	3.49	+.21	6.72	6.70	-.02	48	40	93.3

TABLE II

RECOVERY OF GELATIN FROM A MIXTURE OF BLOOD AND GELATIN

PROPORTION GELATIN:BLOOD	PER CENT GELATIN PROTEIN			PER CENT PLASMA PROTEIN			PER CENT GELATIN RECOVERED	PER CENT PROTEIN CONTENTS		BLOOD HEMATOCRIT PER CENT RED BLOOD CELLS
	CALCULATED	RECOVERED	DIFFERENCE	CALCULATED	RECOVERED	DIFFERENCE		GELATIN	PLASMA	
1:1	4.66	4.27	-.39	1.82	2.12	+.29	91.7	5.54	6.98	50
1:1	4.41	3.80	-.60	1.80	2.23	+.43	86.2*	6.88	5.02	44.6

*Analysis of washed, hemolyzed red blood cells was negative for gelatin.

and plasma is adequate for biologic purposes, the differences between the expected and the obtained values being within the limits of error of the method. Due to the viscous nature of gelatin, difficulty is encountered in adequately draining pipettes when measuring gelatin. This may in part explain the loss of gelatin in the recovery experiments, since the pipettes containing plasma and gelatin cannot be rinsed out with trichloracetic acid. When blood and gelatin were mixed, the gelatin recovery was 91.7 and 86.2 per cent. Analysis of saline washed blood cells hemolyzed on freezing and thawing (86.2 per cent recovery experiment) was negative for gelatin. These data would indicate that no, or at most insignificant, amounts of gelatin entered the red blood cells.

After the writing of this paper the work of Little and Wells appeared.[6] These authors employed sodium tungstate as gelatin precipitant and 10 per cent trichloroacetic acid as plasma protein precipitant. No recovery data were supplied.

SUMMARY

1. A technique applying known methods has been described for the determination of gelatin in a mixture of gelatin and plasma.

2. Recovery experiments indicate the method suitable for biologic purposes.

3. The technique described is also applicable to urine and gelatin analysis.

REFERENCES

1. Waters, E. T.: A Comparison of Isinglass and Gelatin as Blood Substitutes, Canad. M. A. J. 45: 395, 1941.
2. Hiller, A., and Van Slyke, D. D.: A Study of Certain Protein Precipitants, J. Biol. Chem. 53: 253, 1922.
3. Ma, T. S., and Zuazaga, G.: Micro-Kjeldahl Determination of Nitrogen. A New Indicator and an Improved Rapid Method, Indust. & Engin. Chem. 14: 280, 1942.
4. Folin, O., and Denis, W.: Nitrogen Determination by Direct Nesslerization, J. Biol. Chem. 26: 473, 1916.
5. Janota, M., Necheles, H., Weston, R. E., Weissman, V., and Levinson, S. O.: Gelatin Infusion in Hemorrhagic Shock, Exp. Med. and Surg. In Press.
6. Little, S. M., and Wells, H. S.: Capillary Permeability to Intravenously Administered Gelatin, Am. J. Physiol. 138: 495, 1943.

MEDICAL ILLUSTRATION

THE CONTROL OF EXPANSION AND CONTRACTION OF RUBBER CASTS*

CARL DAME CLARKE, AND FREDERICK MATTHEW STINER, WASHINGTON, D. C.

SHRINKAGE of rubber prostheses of facial and body members has presented a definite problem. Rubber shrinks slightly on setting (coagulation) and drying. This shrinkage may be great or infinitesimal. Nevertheless, it may be sufficient to cause an improper fit of the artificial part to the human tissues. Therefore the following experiments were carried out in an effort to overcome this difficulty. It was found possible to expand a rubber cast to more than three times its original surface area or slightly less than two diameters by soaking it in benzine. Likewise, it was discovered how to reduce or shrink it to a fraction of its original size. The ability to reduce the size has its place in art and medicine as well as the need for enlarging to offset shrinkage. A practical example of where shrinkage may be a necessity rather than a fault is when a rubber hand is to be made of a proper size to fit a particular individual who has lost his hand. These hands are invariably molded from a second individual (donor) for supplying a cast to the first (recipient).

Both expansion and contraction must take place without an appreciable loss of detail. Therefore, coins having a wealth of detail were chosen for these experiments. Indeed, the microscopic writing on one coin (Figs. 1 and 2) became plainly visible to the unaided eye on enlarging. It was found that in the expansion of a prosthesis the distortion often took place in proportion to the thickness of the rubber. The thinner parts became distorted more rapidly. However, this was offset in the drying of the expanded rubber to a greater extent than had taken place during the expansion. As a result the prosthesis assumed an accurate but enlarged pattern that was satisfactory for recasting into a final prosthesis for use on the patient.

Briefly, the expansion is accomplished by soaking the vulcanized rubber pattern in benzine until it has expanded to a degree slightly above that which is desired. It is then allowed to shrink somewhat to assume correct but enlarged proportions. The pattern is then used for plaster molding for the final rubber prosthesis or cast.

Expansion and contraction are also governed by the amount of filler (clay) in the rubber. The more filler, the greater the contraction. However, expansion takes place to a lesser degree if the cast is filled.

According to the record kept of experiments, the following results in expansion were noted. These experiments were done on rubber casts from plaster molds.

---

*From the Army Medical Museum.

Received for publication, March 1, 1943.

EXPERIMENT 1

Subject: Coin.

Materials: Vultex H-235 2 parts; Dixie clay 1 part

Expanding agent: Benzine

Minutes	Size in mm.	Expansion gain in mm.
0	40	0
45	55	15
65	58	18
80	60	20

This cast reached its maximum expansion of 60 mm. in 80 minutes.

EXPERIMENT 2

Subject: Coin.

Material: Vultex F-293 2 parts; Water 1 part

Expanding agent: Benzine

Minutes	Size in mm.	Expansion gain in mm.
0	40	0
15	60	20
60	70	30

From this experiment it will be noticed that the unfilled cast expanded more rapidly than the filled cast. In 15 minutes it reached 60 mm. in diameter, a 20 mm. gain. It reached its maximum expansion of 70 mm. in 60 minutes.

EXPERIMENT 3

Subject: Coin.

Material: Vultex H-235 2 parts; Dixie clay 1 part

Expanding agent: Benzine

Minutes	Size in mm.	Expansion gain in mm.
0	40	0
5	42	2
10	44	4
15	46	6
30	50	10
45	53	13
60	60	20
225	60	20

This cast reached its maximum expansion in 60 minutes. However, soaking was continued to see whether disintegration would take place. This became apparent as soaking continued over exceptionally long periods of time. Vulcanization plays a large part in the degree of disintegration. Unvulcanized casts disintegrate rapidly. The well-vulcanized cast holds up after prolonged soaking in benzine.

EXPERIMENT 4

Subject: Coin.

Material: Vultex F-293

Expanding agent: Benzine

Minutes	Size in mm.	Expansion gain in mm.
0	40	0
5	43	3
10	46	6
15	46.5	6.5
30	49	9
45	52	12
60	55	15
75	57	17
90	62	22

This cast was slightly thicker than the cast used in Experiment 2, therefore expansion took place more slowly.

EXPERIMENT 5

Subject: Prosthetic Nose.

Material: Vultex H-235 2 parts; Dixie clay 1 part

Expanding agent: Benzine

Minutes	Size in mm.	Expansion gain in mm.
0	74 high, 53 wide	0 high, 0 wide
21	82 high, 62 wide	8 high, 9 wide
45	85 high, 65 wide	11 high, 12 wide

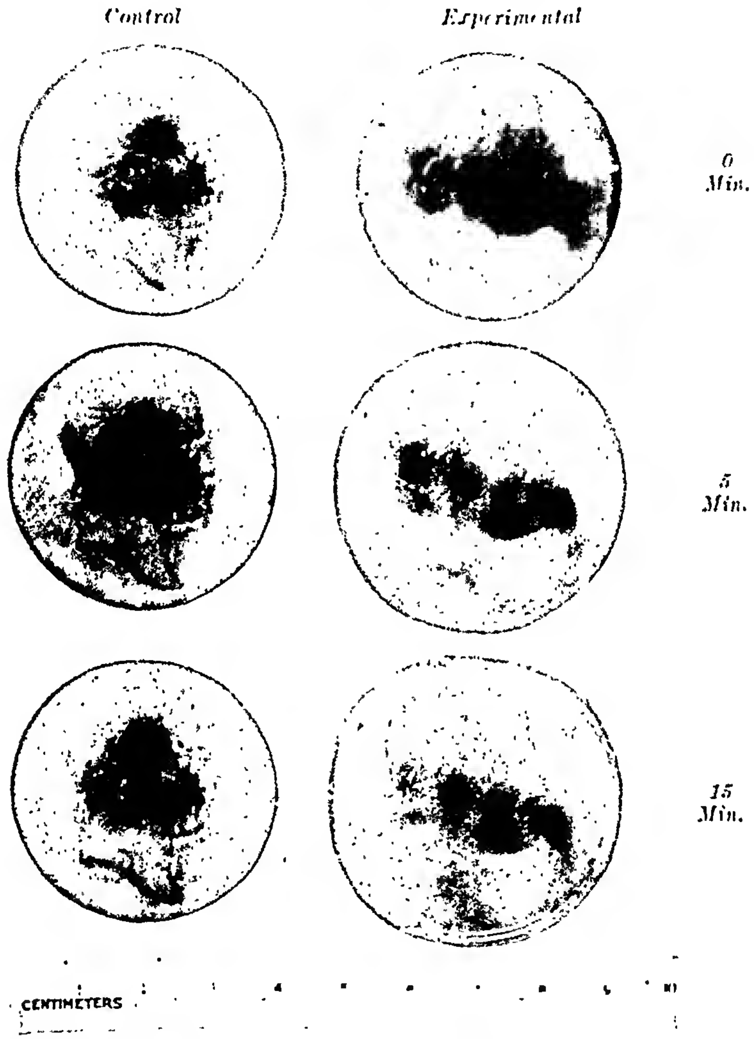


Fig. 1.—The control and experimental of vulcanized latex containing no filler. It will be noticed that after 15 minutes of soaking in benzine distortion begins to appear.

After 45 minutes in the benzine distortion began to take place, so the prosthesis was removed from the benzine. On examination it was found that expansion had taken place rapidly in the thin area. When the prosthesis was allowed to stand in the air it was found that on evaporation of the benzine shrinkage took place more rapidly in the thin areas than in the thick ones. Therefore, the model shrank back to a true ratio in the proportionate areas but there was an enlargement throughout the entire cast. The photograph in Fig. 3 was taken five minutes after removal from the benzine. It shows clearly the proportional

enlargement that is possible. This enlargement is greater than is necessary for prosthetic appliances because the natural shrinkage of rubber is not sufficient to offset such an enlargement. Although it may be necessary to enlarge prosthetic hands and arms beyond the actual size of the donor's hand to provide the

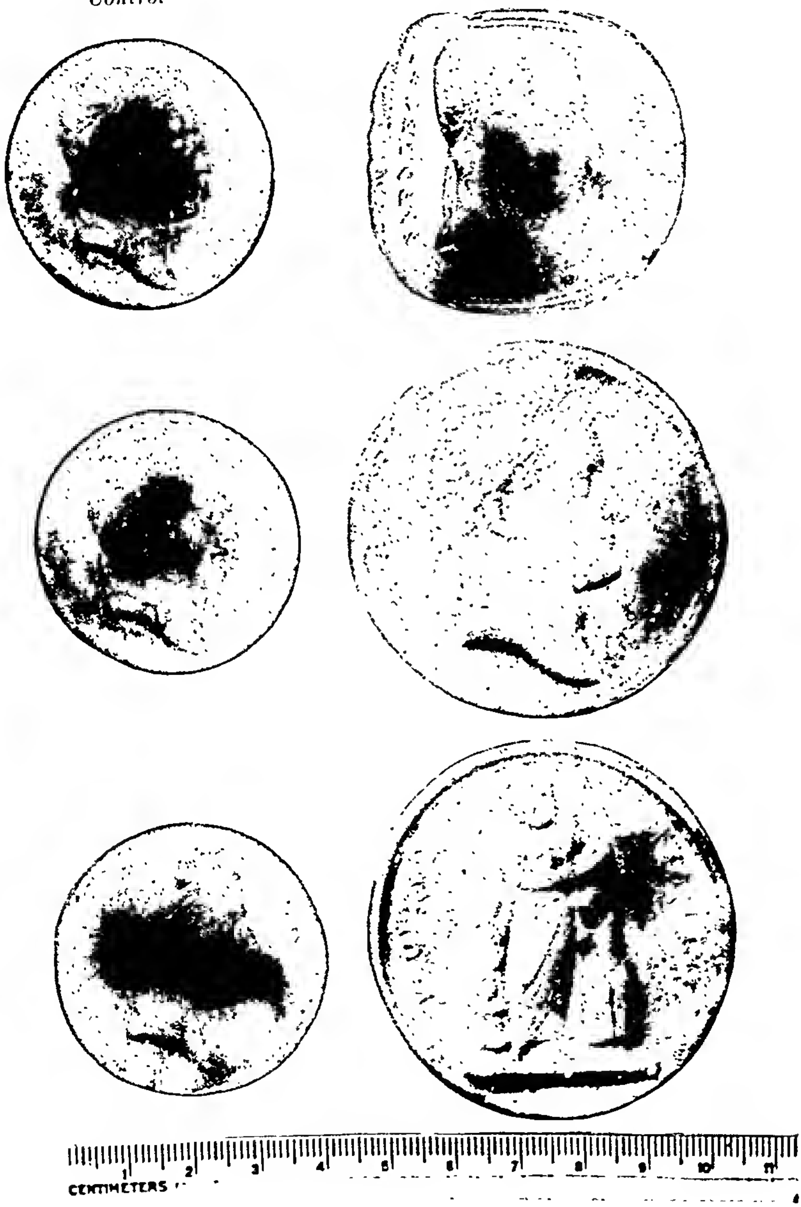


Fig. 2.—This is a continuation of the illustration seen in Fig. 1. Distortion has taken place rapidly in 15 minutes of soaking. At the end of 90 minutes the experimental has flattened without distortion. The bottom picture shows the reverse side of the coin.

correct size for the recipient, it is not likely that an enlargement of such proportions would be necessary in many cases. However, greater enlargements in true ratio and proportions are possible.

As mentioned previously, a distorted enlargement of greater proportions

was obtained in 45 minutes' soaking in benzine. Five minutes after it was removed from the benzine the prosthesis shrank to true but still enlarged proportions to the control. As the benzine continued to evaporate the prosthesis became smaller and eventually returned to the same dimensions as the control. This took about two hours. At any point during this shrinkage the model could have been remolded to obtain a permanent prosthesis of enlarged proportions that would not shrink appreciably regardless of time.

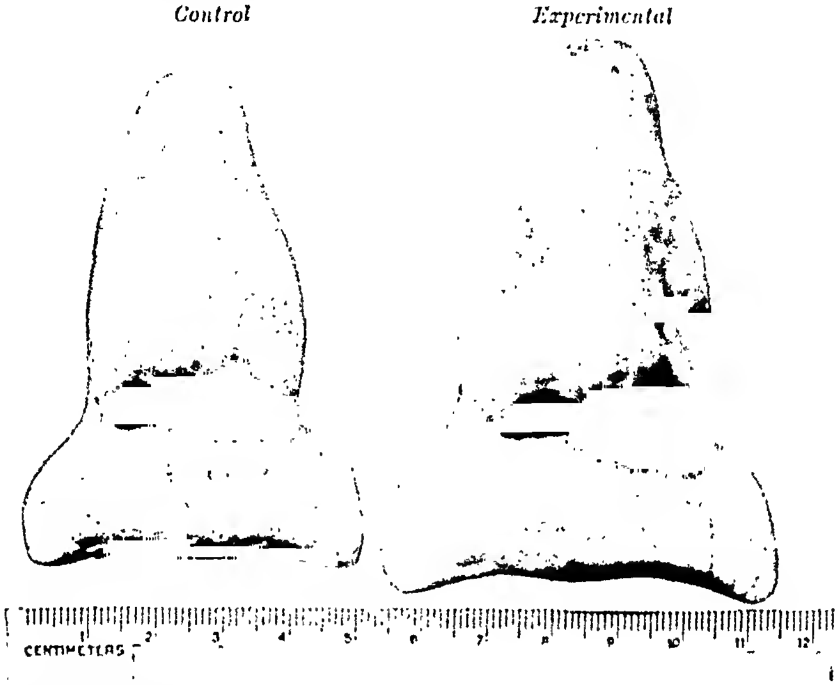


Fig. 3.—Rough casts of filled rubber prosthesis showing expansion in true proportions. This has expanded more than necessary, since it is only essential to offset normal shrinkage for this type of prosthesis. Filled rubber shrinks about 2 mm. to 40 mm.

Distortion.—Upon further investigation it was found that the experimental eventually assumed true proportions when it reached the saturation point during soaking in the benzine. Enlargement of nearly two diameters or roughly three times in area was obtained. The more filler used in the rubber latex the smaller was the enlargement. Nevertheless, this had little effect on distortion. As the enlarging process advanced the model lost physical strength but it always possessed enough toughness to be molded in plaster at any time during the soaking in benzine. In prosthetic hands the expansion can be held permanent for a longer period of time or shrinkage prevented by filling the rubber shell with a microcrystalline wax. This wax is pliable and permits the fingers to be moved in any position desired before molding. A one-piece plaster mold is then made of the rubber hand. After the plaster mold has set the wax is melted out. The collapsible rubber pattern shrinks and is finally pulled from the mold. This one-piece mold is used for pouring duplicate rubber hands to be attached to the patient. In this way prostheses without seam lines are obtained. This technique will be described more fully in a future article.

Accelerated Shrinkage.—Contrary to common belief it should be realized that rubber can be cast in practically any type of mold. This includes molds of agar, plaster, wood, cement, terra cotta, glass, rubber, and synthetic plastics.

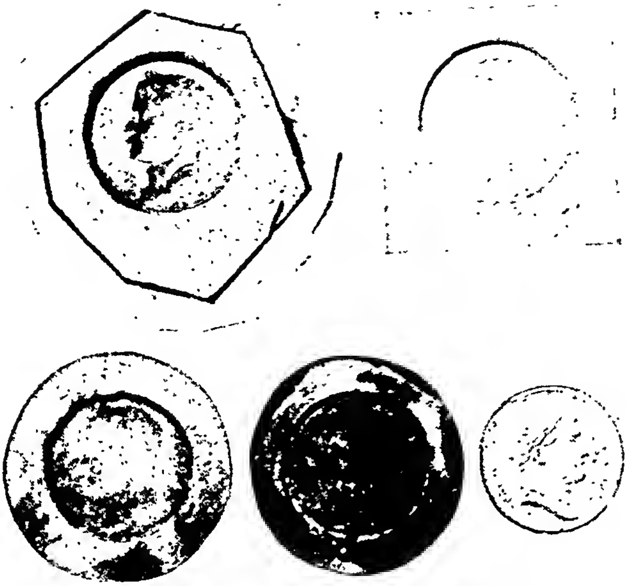


Fig. 4.—Rubber casts can be made from many types of molds. *Upper left:* An agar mold. *Upper right:* A plaster mold. *Lower left:* A wax mold. *Center:* A rubber mold. The small impression at the lower right is a rubber cast.

Fig. 5.—*Top left:* Original coin. *Top right:* First cast from a plaster mold. This cast contained no filler. It was used as a control. *Lower left:* The first cast from an agar mold showing a reduction. *Center:* A second recasting from an agar mold showing further reduction. *Lower right:* A third recasting showing a still further reduction. These recastings were of filled rubber and photograph better than the unfilled rubber which was nearly transparent.

In fact, the problem has never been with the molding material, but new separating mediums had to be devised to suit the mold. Rubber adheres to rubber, therefore soap and a salt acting as a coagulating agent for rubber had to be used on the mold. Rubber will not stick to agar. However, a coagulating salt had to be added to the agar mix to coagulate the rubber latex on the surface of the agar to build up a sufficient thickness of rubber to make the cast of practical use. The subject of separating and coagulating agents also will be discussed further in a future article. This subject has been considered in some detail in the book *Molding and Casting.*[1]

Shrinkage takes place more readily in casts that dry from the side next to the mold. Those that dry from the hollow side of the cast only, shrink less. For example, when rubber is poured into a dry plaster mold, water is absorbed from the latex mix into the porous mold. If rubber is poured into an agar, a rubber or synthetic plastic mold which is nonabsorbent, a rubber coating or layer must be built upon the mold. The excess then is poured out. This coagulated layer of rubber must dry from the hollow side. Furthermore, after coagulating it must not adhere to the surface of the mold. Both the inner and outer surfaces are exposed to the air and shrinkage takes place rapidly. This shrinkage can be offset to some extent by pouring over the coagulated and partially dried rubber layer a microcrystalline wax which presses the rubber back to the mold surface.

If a filled rubber latex is poured into an agar mold, proportional contraction or shrinkage readily occurs on the drying of the rubber. An agar mold will also quickly contract on drying. Here considerable distortion takes place in the mold. This distortion can be prevented by the addition of sorbitol to the agar mix. Shrinkage follows but it is not distorted contraction. In the first casting of a filled rubber latex positive in an agar mold containing no sorbitol a proportional shrinkage of 5 to 40 millimeters occurs, thus producing a cast of 35 millimeters in diameter. The second casting from a second agar mold of the first casting shrinks from 35 to 30 millimeters, and the third casting shrinks to 27 millimeters. Therefore, three castings into agar molds produce a proportional shrinkage of 13 millimeters. As a result, the final cast is reduced to nearly half a diameter of the original pattern. The same process can be continued without appreciable loss of detail.

Prinz[2] gives the following formula for reducing the size of plaster of Paris casts:

Alcohol	1 part by weight
Water	2 parts by weight
Plaster of Paris	enough to suit

The cast is allowed to dry for a few days in a warm place. After the alcohol has been evaporated completely, the finished cast will be about 1/25th smaller in all directions than the original model from which the impression was made. By repeating the process any desired reduction may be obtained.

The reduction of only 1/25th in this case is so small that the experiment was not done. At this rate many castings would be necessary to obtain the 1/8th reduction possible with one casting of rubber into agar. However, from a research standpoint Prinz's method may prove of value.

[1]Clarke, Carl D.: Molding and Casting, Baltimore, 1938, John D. Lucas Co.
[2]Prinz, Herman: Dental Formulary, Lea & Febiger, Philadelphia, 1930.

BOOK NOTICES

Fundamentals of Immunology*

UNTIL within the last decade medical writers have approached the subject of immunology chiefly through the medium of bacteriology. Most of the leading texts on the subject have dealt with bacteriology and immunology. Recent advances in the study of the nature of antibodies and the mechanism of their reaction with foreign cellular and noncellular proteins have been made especially through the medium of protein chemistry and physical chemistry. Dr. Boyd's book, "Fundamentals of Immunology," presents an adequate discussion of recent advances in this new phase of the study. It may be highly recommended as a reference volume on the mechanism of antigens, antibodies, and their interactions, and it is so constructed that it will also serve as a textbook and laboratory manual. The discussion of anaphylaxis is rather brief. That of clinical allergy is decidedly superficial, but this phase of the discussion was not the purpose of the book.

The discussion of antigen-antibody reactions includes study of blood groups, complement and complement fixation, and immune reactions to bacteria, viruses, and parasites.

Physiological Regulations†

DOCTOR Adolph's monograph, *Physiological Regulations,* deals chiefly with the study of the mechanism maintaining water balance in experimental animals and in man. It is highly technical and should be of chief interest to physiologists and biologists.

Familial Nonreaginic Food-Allergy‡

DR. COCA, like many other allergists, has long been conscious of the inadequacy of skin tests with food extracts. His new book, *Familial Nonreaginic Food Allergy,* describes a new approach to the study of allergenic foods. Some food extracts produce positive skin reactions which are truly reaginic. Others appear to give false negative reactions. Dr. Coca believes that these are nonreaginic. His experience has been that this type of food, when eaten by one who is sensitized to it, produces an increase in the pulse rate which can be used diagnostically.

Dr. Coca documents his thesis with detailed presentation of case records. He describes the technique of diagnostic study in detail, and outlines the program of practical management.

Irrespective of whether the author's theory concerning the effect of foods on the pulse rate will be substantiated by others, the volume covers the careful investigations of a leader in the field of allergy and will be of interest not only to allergists, but to internists and others.

Psychosomatic Medicine§

PSYCHOSOMATIC medicine may be termed the newest specialty in the field of internal medicine and psychiatry. The psychiatrist has in the past dealt with psychotics, many of whom are incurable, but has recognized the field of functional nervous disorders in which

*Fundamentals of Immunology. By William C. Boyd, Ph.D., Associate Professor of Biochemistry, Boston University, School of Medicine; Associate Member, Evans Memorial, Massachusetts Memorial Hospitals, Boston, Mass. With 45 *Illustrations*. Cloth, 446 pages, $5.50. Interscience Publishers, Inc., New York, N. Y., 1943.

†Physiological Regulations. By Edward F. Adolph, Associate Professor of Physiology in the University of Rochester. Cloth, 502 pages, $7.50. The Jaques Cattell Press, Lancaster, Pa., 1943.

‡Familial Nonreaginic Food-Allergy. By Arthur F. Coca, M.D., Medical Director, Lederle Laboratories. Cloth, 160 pages, $3.00. Charles C Thomas, Publisher, Springfield, Ill., and Baltimore, Md., 1943.

§Psychosomatic Medicine. The Clinical Application of Psychopathology to General Medical Problems. By Edward Weiss, M.D., Professor of Clinical Medicine, Temple University Medical School, P[illegible] [illegible]urgeon English, M.D., Professor of Psychiatry, Temple University Medic[illegible] Cloth, 687 pages, $8.00. W. B. Saunders Company, Philadelphia and [illegible]

supervised orientation may relieve the patient. It is in this group that the Freudian procedures of psychoanalysis and psychotherapy, used in the broad sense, are often helpful. The internist, on the other hand, treats many cases with organic disease in which he realizes that emotional factors play a part but which he is unable to study adequately because of too limited experience in psychotherapy. Collaborative efforts by the psychiatrist, or psychologist, and the internist may be expected to solve many of these patients' problems. Psychosomatic medicine may be loosely described as the treatment of emotional factors which color the symptomatology of somatic or organic disease.

The volume *Psychosomatic Medicine* results from the collaborative effort of an internist and a psychiatrist. The first two chapters deal with presentation of the problem, with description of illustrative cases. Reading these chapters, one will agree enthusiastically with the authors as to the need for a new method of treatment for such cases and is likely to conclude that the procedure should be very simple, just a matter of horse sense. The last four chapters describe the technique of psychosomatic study and treatment in detail. After reading them, one realizes that this is a highly technical field requiring extensive training, if the therapist wishes to do good rather than harm. Although psychosomatic practice will necessarily develop into a strict specialty, every physician practicing medicine should be well acquainted with the problems and methods in the field. This volume fills this need adequately. In it one finds discussions of the psychosomatic factors in cardiovascular disease, disorders of the gastrointestinal system, the endocrine system, the respiratory system, allergic diseases, the central nervous system, the genitourinary system, and disorders of sexual function. There is also a chapter on military medicine.

Man in Structure and Function*

MAN *in Structure and Function* is a two-volume semipopular discussion of human anatomy and physiology written by a German physician and translated by Dr. George Rosen. The text is free enough from technical terminology and complicated verbiage so that a well-educated layman, not averse to making the acquaintance of some new words, can read it with understanding. The fascinating part of the book, however, is its illustrations. These might be termed "inspired." They illustrate statements concerning the structure and especially the function of the body in the form of comparisons with familiar objects and experiences which all readers can understand with no difficulty at all. There are 461 of them. These volumes are worth their price for the illustrations alone.

*Man in Structure and Function. Volumes I and II. Translated from the German and edited by George Rosen, M.D. Cloth; Vol. I, 311 pages; Vol. II, 712 pages; $10.00. Alfred A. Knopf, New York, 1943.

COMMUNICATION

The first Women's Army Auxiliary Corps Hospital Unit, organized to replace able-bodied men in laboratories and hospitals of Army posts and stations, is now serving with the Army at Station Hospital, Fort Oglethorpe, Ga. This group of medical, surgical, and laboratory technicians, x-ray technicians, dental assistants, pharmacists, medical stenographers, clerks, and typists is expected to be the forerunner of other WAAC hospital units to be organized and sent out to Army posts. WAACs are also serving as technicians, hospital mess sergeants, cooks, and chauffeurs.

The Journal of
Laboratory and Clinical
Medicine

VOL. 28 AUGUST, 1943 NO. 11

PROGRESS

NUTRITION IN THE WAR

FRANKLIN C. BING, PH.D.,* CHICAGO, ILL.

DURING the first World War emphasis was on food. Now it is on nutrition, for this is the science concerned with the proper use of foods for the development and maintenance of healthy, well-nourished bodies. A great deal of factual information has been obtained in laboratories and clinics on the composition of foods and on the dietary requirements in health and in disease. Work along some of these lines has been intensified because it has been recognized that additional information was needed without delay for the handling of wartime problems. The principal activity, however, has been in the application of nutritional knowledge gained through fundamental research. This field of applied nutrition has required the active participation of workers in many other fields. It is the purpose of the present article to describe some of the current important aspects of applied nutrition and to mention also some of the problems that are being investigated at the present time.

SPECIAL PROBLEMS INTRODUCED BY THE WAR

Feeding the Army and the Navy.—"An adequate dietary," Colonel Paul E. Howe[1] has written, "is important for the soldier's health, morale, and effectiveness." In the Army, the immediate responsibility for procurement, distribution, and preparation of foods rests with the Quartermaster Corps, while the supervision of the diet of the soldier is the responsibility of the medical officers in conjunction with the officers of the line. The effort has been to have the American soldiers the best fed troops in the world, and, from reports of

*Director of the American Institute of Baking. Formerly Secretary of the Council on Foods and Nutrition of the American Medical Association.

Received for publication, June 29, 1943.

Colonel Howe, in charge of nutrition activities for the medical department, this goal has been attained for men in training or in service in the United States. The records show clearly that the individual soldier is receiving all of the dietary essentials in quantities exceeding the recommended daily allowances of the Food and Nutrition Board of the National Research Council.[2] It is gratifying to know that the tremendous problem of feeding the American troops is being handled so successfully.

Reports on the feeding of troops in overseas service and in combat areas probably will not be forthcoming until after the war, but there is every reason to believe that the men are well taken care of nutritionally. Rations for use overseas have been described by Wodicka.[3] Considerable interest has developed in the combat ration known as Field Ration K, developed originally in response to a request from the parachute troops, but of such merit that it has proved to be of general usefulness as an emergency ration. Three different units are provided in durable containers which together weigh about two pounds. The foods consist of different types of biscuit, canned meat with cheese, and a beverage concentrate. Chewing gum and cigarettes are also included. The meat, confectionery and beverage concentrates are different for each of the three meals. The nutritive value of these three meals has been designed to meet the requirements of the men. In the development of this ration, the Subsistence Research Laboratory of the Quartermaster Corps, located in Chicago and under the direction of Colonel R. A. Isker, has shown considerable skill and resourcefulness.

In the development of other special rations, a considerable amount of investigation is still under way, and, naturally, the results of such studies have not been published as yet. In this work, experts and officials of the Army, Navy, Marine Corps, Coast Guard, and Merchant Marine work in close cooperation with the Committee on Medical Nutrition of the National Research Council, the chairman of which is Dr. James S. McLester. This Committee is able to supply or obtain quickly the best available opinion on scientific questions and to plan tests for developing the answers to problems that need experimental investigation. Frequently these investigations are made in university laboratories under contract with the Office of Scientific Research and Development. The Navy recently has developed a Naval Medical Research Center located at Bethesda, Maryland, where nutritional and other questions related to the many special problems confronting the Navy can be studied. The scientific director of the Naval research laboratories is Dr. A. C. Ivy.

The emphasis on foods for combat troops is on keeping quality, compactness for ease in shipping, palatability, for no food is of any value unless the men will eat it, and on nutritional value, because it is recognized that if the foods do not provide the nutritional requirements of the men, recourse will have to be had to other methods of meeting these requirements. Industry has cooperated wholeheartedly and importantly with these newer developments, and, in the production of dehydrated foods especially, and in the perfection of proper packaging for special conditions, great advances have been made. Undoubtedly some of the newer developments will be useful to the civilian population after the war.

Much attention has been devoted to the requirements of foods for use by shipwrecked personnel, by troops in desert or jungle areas, or wherever an emer-

gency ration is needed in situations requiring conservation of the available supply of water. It is well known that the ingestion of protein foods and of inorganic salts subsequently involves the excretion of urea and salts in aqueous solution through the kidneys, thus depriving the body of some of its water. It would seem desirable therefore to reduce the protein and mineral content of these emergency rations to a minimum, or to avoid these foods entirely in favor of carbohydrate and fat. The use of candy containing citric acid, a stimulant for salivary secretion, has been developed. It would seem, however, that the formulation of suitable dietary schemes for use under such conditions is one which might well attract many investigators who are not now engaged in war problems. Is a man floating in a rubber lifeboat on a tropical sea better off eating nothing rather than eating fish which he may be able to catch? What should be the nature of the emergency foods provided? These are important nutritional questions.

There are many problems concerned with the feeding of men who are exposed to unusual environmental conditions, as in service on submarines or in airplanes. Bierman[4] has discussed the problems of aviation medicine concerned with air sickness, obesity, and the expansion of gases that form in the intestinal tract at higher altitudes. The condition of air sickness develops as a rule from acceleration during flight. It is considered to be a functional neurosis characterized by vomiting, nausea, apprehension, faintness, sweating, and sometimes prostration, and it may be precipitated or aggravated by the sight or odor of food. Men appear to be specially prone to develop air sickness on an empty stomach or after a heavy meal, and therefore Bierman has suggested that the meals should be light and served at four-hour intervals. Obesity in the aviator is undesirable, because fat affords an excellent storage for nitrogen and hence appears to aggravate the development of "bends" at high altitudes. The expansion of intestinal gases at high altitudes may cause intense discomfort and cramps that may persist for as long as twenty-four hours after returning to sea level. The elimination of gas-containing foods and beverages and those foods which are known to be particularly prone to develop flatus helps to avoid this difficulty, as does the feeding of frequent light meals. There is evidence that vitamin C is of considerable importance in enabling men to fly at high altitudes. The relationship of vitamin A to visual acuity, especially in dim light, is less clearly defined, but it is universally admitted that the rations of all military personnel should be thoroughly adequate with respect to all the vitamins and other dietary essentials, and a high content of vitamin A is desirable.

The value of the administration of additional vitamins when the diet itself is adequate with respect to these factors has been experimentally studied by Keys,[5] with negative results. On the other hand, there is accumulating evidence to show that the ability to perform work is decreased on low thiamine diets and improved following the administration of thiamine under these conditions. Thus on diets providing about 0.33 mg. of thiamine and 0.5 mg. of riboflavin daily, Foltz, Barborka, and Ivy[6] showed clearly that the work output was decreased and the subjects complained of lack of vigor, anorexia, and leg pain. When the deficient diet was supplemented with suitable quantities of yeast concentrate, providing thiamine, riboflavin, and other members of the B complex, there was a

prompt increase in the work output and a disappearance of the symptoms. At the Fatigue Laboratory at *Harvard University*, Egaña,[7] Johnson,[8] and their co-workers have also observed a close relationship between an adequate amount of thiamine and the ability to perform muscular work.

Problems Concerning the Feeding of Civilians in the United States.—The problem of meeting the civilian requirements for good nutrition is much more involved than the problem of feeding the armed forces. An excellent discussion of food supplies and public policy, food production and distribution is provided in the series of articles published under the title of "Nutrition and Food Supply: The War and After" in the Annals of the American Academy of Political and Social Science for January, 1943. Agencies of government have been established with the view of guiding agricultural production of foods and the distribution of foods in the wisest possible manner. The complexity of the problems involved may be illustrated by the situation which developed with corn in June, 1943. Because of prevailing ceiling prices, an editorial in the Southwestern Miller[9] states that farmers could anticipate obtaining about $1.50 for the pork produced from feeding about $1.00 worth of corn to hogs. As a consequence there developed a shortage of corn for human food purposes and for industrial needs and a tremendous increase in the hog population. The products of corn are needed not only for the manufacture of corn meal, cornstarch, certain breakfast cereal foods, and other foods such as corn sirup and dextrose, but also for the growth of significant amounts of the yeast used in the making of bread and for materials used in the manufacture of explosives, aluminum, magnesium, bronze, steel, and other war products. By the middle of June the War Food Administration had taken emergency steps to help relieve the situation and to enable essential war production to continue.

The rationing of foods has been developed to permit a more equitable distribution of foods in short supply. These include meat and butter and other fats, the supplies of which are being used in large part for military and lend-lease purposes, and sugar and coffee which must be imported. Canned foods have been rationed because they also are being used for military and lend-lease purposes and because any marked increase in production would involve the use of strategic materials and manpower. The reason for requiring ration coupons for evaporated milk is said to be in order to place this important food under control. Serving in an advisory capacity to help assure that rationing regulations of the Office of Price Administration will be in the interests of continued health of the people is the Civilian Food Requirements Branch of the Food Distribution Administration, of which Dr. Russell M. Wilder is Chief.

In the rationing of foods for civilians, consideration must be given to nonfood uses, the requirements of food industries, restaurants, and institutions, and the requirements of consumers. The general policy has been to keep rationing regulations as simple as possible. There is a trend also to give preference in the materials allocated to industrial users to those manufactured foods where foods in short supply help further the use of those articles the supply of which is more abundant. In the allocation of relatively scarce items to industrial food processors, some consideration has been given also to the nutritional importance of the finished products.

People ordinarily learn about the nutritional value of foods from various sources, among which is advertising. Guidance of food advertising along educational lines long has been a concern of the Council on Foods and Nutrition of the American Medical Association. Realizing the importance of such work, the government has established a plan of cooperation with advertisers of food products known as the War Nutrition Program under the supervision of Drs. M. L. Wilson and W. H. Sebrell of the Nutrition Division of the Office of Defense, Health and Welfare Services of the Federal Security Agency, the administrator of which is the Honorable Paul V. McNutt. Early in 1943 the Nutrition Division was transferred to the U. S. Department of Agriculture and is now known as the Nutrition and Food Conservation Branch of the Food Distribution Administration. There has been a commendable trend on the part of food manufacturers and distributors to show in their advertising how the food article which they are selling fits into the scheme of an adequate diet for health. A new chart recently has been developed which describes seven basic food groups that should be included in the diet each day in the interests of good health. Thus advertisers of foods, in contributing to the National Nutrition Program, will be presenting the same basic information about the characteristics of an adquate diet.

Feeding Industrial Workers.—Recognition of the importance of the problem of feeding industrial workers is evidenced by the fact that at least three important committees have been established for the consideration of questions that arise in this field. The Food and Nutrition Board of the National Research Council has a subcommittee on the Nutrition of the Industrial Worker, and the Councils on Foods and Nutrition and on Industrial Health of the American Medical Association have formed a cooperative committee on Nutrition in Industry. The Office of Defense, Health, and Welfare Services maintains a division on Nutrition in Industry, which functions largely through the regional representatives and the State nutrition committees that have been established under the supervision of this agency. Close cooperation exists between this department and the Industrial Hygiene Division of the United States Public Health Service. A number of informative reports have been published and several research projects have been organized for the purpose of answering questions in this field. As Goodhart[10] has pointed out, a great burden of the work must be borne by local nutrition committees. The industrial nurse and the industrial physician often can help immeasurably in solving the immediate problems of an industry.

Surveys by Wiehl[11] of the diets selected by aircraft workers in California have shown the choice of foods to be exceedingly poor and it is reported that the condition has become worse rather than better. She found that if one assumed values equal to two-thirds of the recommended dietary allowances of the Food and Nutrition Board to be a minimum value for adequacy, the diets selected by these workers were deficient as follows: about 15 per cent were low in vitamin A, 25 per cent in calcium, over 40 per cent in riboflavin and in ascorbic acid. Medical appraisal of the nutritional status of such workers has afforded a comparable picture of deficiency, certainly a condition that is not conducive to continued maximum output of war materials. The principal weapon for overcoming such deficiency would appear to be education, but in the case of the

industrial worker, it is possible, and no doubt essential, to do more than that. It has been pointed out that in many plants, particularly the smaller ones, there are no facilities whatever for providing the workers with lunch or with mid-shift meals. Indeed the tendency is to keep the amount of time for eating to a minimum. The carrying of lunches or other packages into some of the larger war plants is prohibited because there are inadequate facilities for inspecting the contents of such packages. The manner in which some firms have met these problems and a discussion of other current nutritional activities in industrial establishments has been provided by Cowgill.[12]

Considerable interest has been aroused in connection with the question of giving all industrial workers vitamin preparations. In reviewing the evidence on this subject, a Joint Committee of the Council on Foods and Nutrition and of the Council on Industrial Health of the American Medical Association expressed the view that such indiscriminate administration was economically unwise, nutritionally unsound, and therapeutically irrational.[13] This Committee believes that much of the vitamins so administered would be wasted, and it expresses the opinion that vitamins could not correct all possible dietary deficiencies and holds to the conviction that the administration of vitamins is best handled under medical supervision, especially by the practitioner who has had an opportunity to examine the individual patient and thus to ascertain his requirements. More recently the argument has been advanced that the giving of vitamins now is desirable because of food rationing and because the vitamins in themselves will do no harm. Except for vitamin D, large doses of which may produce untoward effects, it is correct that the vitamins will do no harm. On the other hand, it is still questionable whether under the usual conditions contemplated for the administration of vitamins on a mass scale, they would accomplish as much good as would be accomplished by attention to the diet. Further, the supply of vitamin preparations is not inexhaustible, and increased production of thiamine, riboflavin, and ascorbic acid requires equipment, strategically important chemicals, and manpower.

THE NUTRITIONAL STATUS OF THE PEOPLE

A recent report[14] of the Committee on Diagnosis and Pathology of Nutritional Deficiencies, of the Food and Nutrition Board of the National Research Council, entitled "The Prevalence of Inadequate Diets and Deficiency States in the United States Together With a Consideration of Their Significance" asserts that every survey in the past decade has revealed widespread dietary deficiencies in the United States. It has been argued, however, that surveys of food consumption do not necessarily give a true picture of the condition of health of the people, and medical records have been cited to show that the incidence of malnutrition is almost insignificant. A discussion of the discrepancy of these two views has been presented by Jolliffe, McLester, and Sherman,[15] who have given reasons why national mortality tables and hospital admission rates tend to present an overly optimistic picture. Space does not permit a discussion of the results of newer methods of medical appraisement of the nutritional status, particularly because some of the methods are not yet firmly established; but probably every critic would agree that the diet on the whole is not as good as it ought

to be, and could be, and that the work of applying nutritional principles to practical human problems must be continuously carried on. The problem now as before is to teach people the principles of proper eating. There is insufficient evidence to judge the effects of rationing on the general nutritional status of the people in the United States. Some persons believe that the present situation has served to focus attention on the need of selecting foods on a nutritional basis. We are assured also that even with rationing the American dietary is adequate in quality and quantity of nutrients.

Great Britain.—A number of reports have indicated that the nutritional status of the people in Great Britain is, on the average, probably better than it was before the war.[16] If this is correct, it is likely that rationing schemes which guarantee the more equitable distribution of foods have been in large measure responsible. Since early in 1942 all infants, expectant mothers, and small children have been receiving daily some sources of vitamins C and D at least during the winter months. These groups also received extra rations of milk. Arrangements have developed so that people unable to afford these foods are given them just the same.

Although the overall picture is gratifying, it is true that some groups which before the war were better fed are in poorer nutritive condition today because of rationing. Body weight is said to be somewhat below the usual weight. The tuberculosis rate, however, has not changed markedly. There is some evidence that the incidence of anemia has increased, although the iron content of the diet probably is greater than it has been previously. The principal weakness in the British diet today, however, is in its vitamin C content. Although evidence of scurvy has not been observed, the people are living on diets very close to or below the minimum requirement of 30 mg., which is considered to be the amount necessary to prevent scurvy. The recommended daily allowance for the normal adult in the United States is considered to be 75 mg. Steps undertaken to correct this condition have been the encouragement of planting home gardens of green leafy vegetables and the utilization of unusual foods such as rose fruits which are relatively rich in ascorbic acid. In this connection, a recent report of Hunter and Tuba[17] in Canada is of interest. They found that edible products could be made from rose hips available in Canada, and it can be calculated that the expected harvest from Alberta would be sufficient to supply 140 million people (being equal to the population of the United States) with 100 mg. of ascorbic acid daily for a year.

THE OUTLOOK

With all of the attention that is being devoted to the subject, it is not overly optimistic to believe that the allied nations will come out of the wor with their nutritional status unimpaired and perhaps in many instances improved. Evidence of the fact that the nutritional point of view is taking hold is apparent to every close observer. Agricultural economists are beginning to speak occasionally of the nutritional yield of an acre of ground. Programs of agricultural production are laying considerable emphasis on the desirability of agriculture being geared to meet the nutritional requirements of the people. Laboratories have been established for the examination of soils and varieties of plants with

a view to ascertaining the kind of treatment of soils and the varieties of plants to select in order to reap the maximum nutritive value from the crops. Commercial processors of foods are becoming more and more concerned with the nutritive value of their products. Studies are being undertaken with a view to ascertaining the facts and then taking steps to conserve to the highest possible degree the nutritive value of the natural foods from which the processed foods are made. Notable in this connection is the program of the National Canners Association and the Can Manufacturers Institute on the nutritive value of canned foods.[18]

Fortification of foods has been placed on a sound basis,[19] as a result of considerations given by bodies such as the Council on Foods and Nutrition of the American Medical Association and the Food and Nutrition Board of the National Research Council. Of the common staple items there are only four classes of foods to which general approval has been given as vehicles for the administration of vitamins or minerals or both. These are enriched white flour and enriched white bread, which provide additional amounts of thiamine, niacin, and iron and, when supplies become available, of riboflavin, oleomargarine containing added vitamin A, iodized table salt, and vitamin D milk. The most recent of these developments has been the enrichment of flour and bread, and data now are available to show the value of this procedure.[20, 21]

In connection with the fortification of foods, mention should be made of the important work that the Food and Drug Administration of the Federal Security Agency, under the able leadership of Mr. Walter G. Campbell, has been doing. Definitions and standards have been developed for many common foods as well as for the fortified foods already mentioned, thus aiding "in protecting the public against exploitation by programs dictated more by commercial motives than by public interest."

Recently hearings have been concluded for the purpose of receiving evidence necessary to amend the standards for enriched flour and bread in accordance with recommendations of the Food and Nutrition Board of the National Research Council. Students of nutrition will find a study of the records of these hearings to be of great interest. The findings of fact relative to the higher enrichment levels for flour have been published.[21b]

A POSSIBLE WORLD-WIDE NUTRITION PROGRAM

Professor John D. Black[22] of the Department of Economics at Harvard University has written of the importance of nutrition in the postwar years in the following words: "All the knowledge of foods and nutrition that we now have must in due time be turned to the task of restoring order and building up a peace that will not be menaced by hunger and deficiency diseases." This concept of nutrition as a potent factor in harmony among nations in a postwar world was specially emphasized at the United Nations Conference on Food and Agriculture recently concluded at Hot Springs, Virginia. Probably the average person who has read only the newspaper accounts of this Conference is familiar with only two facts that were widely publicized: the fact that newspaper representatives were not admitted to executive sessions, and the report that considerable amounts of whisky were shipped to Hot Springs while the conference was

in progress. It does not seem to be generally known that there were representatives of 44 governments present, 9 of which were occupied by enemy nations. Though none of these delegates had the power to make agreements for the governments which they represented, it would seem likely that the agreements they did make as a result of the discussions at this meeting will be more binding than have been many of the treaties arranged by diplomats. The Conference probably was the first in history for the discussion of postwar planning while a war was in progress. Attention was directed, not to the requirements of individual countries, but to the rights of the individual human being. There were three sections to the Conference, and Section 1, which was concerned with problems of nutrition and public health, adopted a number of resolutions on May 29, 1943. Can anyone read the following account[23] of the first resolution adopted by this Section without realizing that nutrition is recognized as a factor of dominant importance for health?

The United Nations Conference on Food and Agriculture

having reviewed the information submitted by the several delegations on consumption deficiencies and the relation of food to health through the world and being deeply impressed by the dominant role played by adequate food in the reduction of sickness and death rates and the maintenance of health, the Conference

DECLARES:

1. That the first essential of a decent standard of living is the provision to all men of those primary necessities which are required to promote freedom from disease, and for the attainment of good health;
2. That the most fundamental of these necessities is adequate food which should be placed within the reach of all men in all lands within the shortest possible time;
3. That ample evidence has been presented revealing the existence of malnutrition in every country, with its inevitable consequences of preventable ill health; and

RECOMMENDS:

That the governments and authorities here represented:

(a) Immediately undertake the task of increasing the food resources and improving the diets of their people in accordance with the principles and objectives outlined in the findings of the Conference, and declare to their own peoples and to other governments and authorities here represented their intention of so doing;

(b) Undertake periodically to report to one another through the permanent organization recommended in Resolution II on the state of their national nutrition and on the steps being taken for its improvement.

It is of special interest to note the desire for immediate action in improving the diets of people and, in this connection, it should be stated that the delegates of the occupied countries were in favor of the resolution but desired that a footnote be added to explain that, obviously, countries in the hands of our enemies could not begin such a program right away.

REFERENCES

1. Howe, Paul E.: The Dietaries of Our Military Forces. Nutrition and Food Supply: The War and After, the Annals of The American Academy of Political and Social Science 225: 72, 1943.

2. McLester, James S.: Feeding the American Soldier, Nutrition Rev. 1: 225, 1943.
3. Wodicka, Virgil O.: Preservation of Foodstuffs—Food Requirements for Overseas Use, Indust. & Engin. Chem. 35: 12, 1943.
4. Bierman, Howard R.: Nutrition in Aviation Medicine, War Med. 3: 1, 1943.
5. Keys, Ancel, and Henschel, Austin F.: Vitamin Supplementation of U. S. Army Rations in Relation to Fatigue and the Ability to Do Muscular Work, J. Nutrition 23: 259, 1942.
6. Foltz, E. E., Barborka, Clifford J., and Ivy, A. C.: Influence of Diet Deficiency in the Vitamin B Complex on Work Output of Trained Subjects, J. A. M. A. 121: 1411, 1943; 122: 717, 1943.
7. Egaña, E., Johnson, R. E., Bloomfield, R., Brouha, L., Meiklejohn, A. O., Whittenberger, J., Darling, R. C., Heath, C., Graybiel, A., and Consolazio, F.: The Effects of a Diet Deficient in the Vitamin B Complex on Sedentary Men, Am. J. Physiol. 137: 731, 1942.
8. Johnson, R. E., Carling, R. C., Forbes, W. H., Brouha, L.: Egaña, E., Graybiel, A.: The Effects of a Diet Deficient in Part of the Vitamin B Complex Upon Men Doing Manual Labor, J. Nutrition 24: 585, 1942.
9. Editorial: The Corn Ceiling Failure, Southwestern Miller 22: 23, 1943.
10. Goodhart, Robert S.: The National Nutrition Program for Industry, J. A. M. A. 121: 823, 1943.
11. Wiehl, Dorothy G.: Diets of a Group of Aircraft Workers in Southern California, Milbank Mem. Fund Quart. 20: 329, 1942.
12. Cowgill, George R.: Current Nutritional Activity in Industry, J. A. M. A. 121: 817, 1943.
13. American Medical Association, Report by Representatives of the Council on Foods and Nutrition and the Council on Industrial Health: Indiscriminate Administration of Vitamins to Workers in Industry, J. A. M. A. 118: 618, 1942.
14. National Research Council, Draft Report by the Committee on Diagnosis and Pathology of Nutrition Deficiencies: The Prevalence of Inadequate Diets and Deficiency States in the United States Together With a Consideration of Their Significance. Unpublished.
15. Jolliffe, Norman, McLester, James S., and Sherman, H. C.: The Prevalence of Malnutrition, J. A. M. A. 118: 944, 1942.
16. Nutritional Status of Great Britain, Nutrition Rev. 1: 112, 1943.
17. Hunter, G., and Tuba, J.: Note on Rose Hips and Evergreens as Sources of Vitamin C, Canad. M. A. J. 48: 30, 1943.
18. Cameron, E. J.: Canning Technology, Indust. & Engin. Chem. 35: 38, 1943.
19. Nelson, E. M., Stiebeling, Hazel K., Gordon, Edgar S., Bing, Franklin C., and Oser, Bernard L.: Symposium on Fortified Food of the American Institute of Nutrition, Federation Proceedings 1: 324, 1942.
20. Lane, Robert L., Johnson, Elisabeth, and Williams, Robert R.: Studies of the Average American Diet, I. Thiamine Content, J. Nutrition 23: 613, 1942.
21. (a) Williams, Ray D., Mason, Harold L., and Wilder, Russell M.: Evaluation of Nutritive Contribution of Enriched White Flour, J. A. M. A. 121: 943, 1943.
(b) Food and Drug Administration: Wheat Flour and Related Products. Amendments to Definitions and Standards of Identity, Federal Register 8: 9115, 1943.
22. Black, John D.: Food: War and Postwar, Nutrition and Food Supply: The War and After, The Annals of the American Academy of Political and Social Science 225: 1, 1943.
23. United Nations Conference on Food and Agriculture, Hot Springs, Va., May 18 to June 3, 1943. Final Act and Section Reports. U. S. Government Printing Office, Washington, 1943.

CLINICAL AND EXPERIMENTAL

THE PRINCIPLES OF PERCUTANEOUS ABSORPTION*

Stephen Rothman, M.D., Chicago, Ill.

Absorptivity of the skin has widespread implications in different branches of medicine such as physiology, pharmacology, toxicology, topical and systemic therapeutics, dermatology, industrial hygiene, balneology, and forensic medicine. Consideration of the fact that it may become important in war medicine also prompts a review of the subject at this time.

Electric Forces Regulating Absorption.[1]—On sending a direct current through the human body by means of metallic electrodes the initial intensity of the current drops considerably within fractions of a second, as if there were a sudden increase in ohmic resistance ("pseudoresistance of the skin"). This phenomenon is due to polarization, i.e., to the generation in the body of a current which flows in a direction opposite to that of the original current. The polarization current is produced in the epidermis exclusively. It fails to appear if the continuity of the epidermis is disrupted. It can be obtained in its full strength in the isolated epidermis.

If a direct current has to pass a membrane in a solution of electrolytes, a new electromotive force is produced, originating from the difference in diffusion-velocity of electrically positive cations and negative anions wandering through the membrane (electric diffusion potentials). Often the membrane is permeable only for either cations or anions, in which case a high potential difference may develop by the "electric double layer" on both sides of the membrane. This is the case with human epidermis which has a negative charge and is impermeable for anions.[2] Consequently aqueous solutions of electrolytes do not penetrate the epidermis.

If the isolated epidermis is used as a diaphragm in a vessel filled with water and a direct current is sent through the water, there is a movement of the water from the anode to the cathode.[3] This movement of water through membranes is called electroendosmosis. Its direction is towards the cathode if the membrane has a negative charge. By addition of salts or acids to the water the epidermis, or any other membrane with a negative charge, becomes discharged, and the movement of water is slowed down or stopped entirely. By addition of alkalies which increase the negative charge the movement is accelerated.[3]

The membrane effect of the epidermis is due not to the stratified structure of the Malpighian layer with its many layers, but to a single membrane being in

*From the Department of Medicine, Section of Dermatology, and Department of Pharmacology, University of Chicago.

This article is based on an earlier review of the subject[1] in which references previous to 1932 are quoted extensively. In this review the references will be restricted to publications of major importance of the period from 1920 to 1941.

Received for publication, January 29, 1943.

immediate contact with the electrolyte solution.[2] The horny layer cannot be regarded as a membrane in the electrophysiologic sense, because it represents a rather rough network of horny lamellae with large holes in it, and therefore is easily permeable for ions as well as for large molecules.[1] The electrical behavior of the skin is not influenced by extreme thinning or thickening of the horny layer. Therefore the seat of the polarization phenomena is to be placed in the transitional layers between cornified and noncornified epithelium (stratum granulosum and stratum lucidum). These layers are responsible for the polarization current ("pseudoresistance") and for the impermeability of the skin to electrolytes.[3] They are called the barrier of skin absorption. Being situated between the strongly acid stratum corneum and the slightly alkaline Malpighian layer, they represent an electric double layer with positive hydrogen ions on one side and negative hydroxyl ions on the other side.[1]

Functional changes alter the electrical behavior. Mechanical, chemical, thermic, and electric stimuli cause a diminution of the skin resistance ("local galvanic reactions").[4] This phenomenon is due to changes in the cell membrane causing increased permeability and decreased polarization currents. This condition can be demonstrated in any inflammatory lesion of the skin if the lesion is due to primary damage of the epidermis. Hyperemia due to vasomotor reflex does not change the resistance.

Misconceptions on Transepidermal Absorption.[1]—The surface of the skin is covered by a greasy layer consisting mainly of waxes and cholesterol esters.[5] The role of this covering as a factor in the impermeability of the skin for water and electrolytes has been overestimated. It is true that it hinders the wetting of the cornified surface, and so delays the penetration of water and aqueous solutions into the horny layer. Waxes and cholesterol esters are miscible with water, however, and therefore after prolonged contact with water, particularly with warm water, the permeation of aqueous solutions into the horny layer becomes possible. The keratin material swells in water as it is grossly seen on palms and soles where the thick horny layers show abnormal wrinkling and a whitish discoloration after a prolonged bath.

Similarly to the role of the greasy cover the role of the horny layer has been misinterpreted as a barrier for percutaneous absorption. Its large pores are permeable not only for water and aqueous solutions but for molecules of any size and even for gross molecule aggregates.

A third misconception is the idea that increased blood flow may facilitate the percutaneous absorption of substances which normally do not penetrate. This belief has arisen from the observation of qualitatively increased permeability in inflammatory lesions. Such increase is due to epidermal injury, however, and not to increased blood flow. If once the barrier of transepidermal absorption in the epidermis is broken through by any injury and a substance has entered the living epidermis, its absorption into the blood stream is no longer prevented. Increased blood flow may accelerate the rate of absorption but does not change the permeability of the skin qualitatively.

Absorption Through Appendages.[1]—The mechanism of percutaneous absorption as a whole becomes rather complicated by the presence in the skin of gland ducts and hair follicles which open on the surface in the form of visible

pores. The hair shaft does not adhere to the follicular wall. The space between is loosely filled with greasy, horny scales and contains air. This space is continuous with the duct of the sebaceous gland which empties the sebum into it. Any substance may penetrate this space and reach the duct of the sebaceous glands, and from there the gland cells. This pathway does not require passage through a stratified epithelium but leads through a canal filled with air to cell membranes, the permeability of which is higher than that of the transitory epidermal cells. Similarly, through the sweat gland ducts any substance may reach the secretory surface of the sweat gland cells after having passed the straight and the convoluted part of the duct. When a substance has reached the secretory cells, it easily enters the blood stream, even though it is true that a "basal membrane" has to be passed before the endothelium of the capillaries is reached.

Water and aqueous solutions do not enter the follicular and glandular pores by mere capillary attraction because of insufficient wetting and because the ducts are filled with air. Under pressure, however, the adherent air may separate and escape in form of bubbles as can be seen during a warm bath. In the case of inunction of ointments, compression of the air in the duct and absorption of the air through gland cells may take place.

Experimental Conditions and Methods.—As has often been emphasized, in experiments on percutaneous absorption the intake of substances through the respiratory tract and through the mucous membranes of mouth, rectum, and vagina has to be carefully avoided. Working with volatile substances, i.e., substances with relatively high vapor pressure, one must be particularly cautious to avoid errors in attributing absorption through the lungs to percutaneous absorption.

To prove that percutaneous absorption of substances foreign to the body has taken place, analytic-chemical methods should be preferred to any other method in demonstrating the presence of these substances in tissues, in the blood, or in the excretions. If, in lack of chemical methods, pharmacologic or toxicologic assays have to be used, such assays must be unequivocally specific for the substance in question. Histochemical methods, often used to prove the permeation of a substance into the skin proper, are sometimes of dubious reliability, and one should be particularly critical in evaluating the results obtained with such methods.

For experiments on the absorption of substances normally present in the body, the procedure of Hediger[6] is the method of choice. With some modifications it has been widely used by Bürgi[7] and his co-workers, with consequent valuable contributions to the subject matter. In this method the solutions to be examined are applied to the skin under glass bells with airtight occlusion, and changes in the concentration of the dissolved substances are estimated at certain time intervals in aliquot parts by quantitative chemical analysis of the fluid above the skin. Decrease of concentration indicates percutaneous absorption of the solute. The time curve of the rate of absorption can also be easily determined by this method.

Absorption of Water.—The bibliography of experiments on water intake through the skin of human beings is old and obsolete. Plethysmographic meas-

urements on a limb enclosed air tight in a water bath for several hours do not show any decrease in the volume of water.[1] It is conceivable that negligibly small amounts of water, within the error margin of the plethysmographic method, may enter the pores after having expelled the air. Practical experience, however, especially experience with the permanent "water bed" of the dermatologists, supports the assumption that the amount of water intake under normal pressure is extremely small, if any. Intake of water through the epidermis certainly does not take place. Whereas the horny layer swells throughout its whole thickness when a limb is kept in water, the living epidermis cells remain microscopically unchanged. It has been assumed that the pH of the cell proteins in the transitory layers between the cornified and noncornified epithelium is identical with the isoelectric point of these proteins which therefore are unable to swell in water.[1]

Absorption of Electrolytes.[1]—It can be stated as a general rule that electrolytes dissolved in water do not penetrate the skin of mammals. This impermeability is fairly well understood from the physicochemical point of view as discussed previously. The theory of pores in the cell membrane based on model experiments[8] furnishes a most satisfactory explanation for the impermeability of the skin for anions. Membranes with extremely fine pores adsorb selectively, depending on the electric charge, either cations or anions, and therefore these "ion sieves" are impermeable for electrolytes. Tiny canals in the cell may correspond with such pores.

The easiest way to demonstrate the impermeability of the skin to aqueous solutions of electrolytes is to apply to the skin salts, which normally are not present in the body, and to determine whether these compounds, if applied to the skin, are excreted with the urine. Salts with nonphysiologic cations, such as salts of lithium, cesium, rubidium, and strontium, were tested in this way. They were applied to the skin in aqueous solutions in the form of baths, sprays, etc., but none of them could be recovered in the urine.[1, 9] The impermeability of the skin for salts containing the physiologically occurring cations sodium and calcium has recently been proved by means of Hediger's method.[10]

Of the anions the iodide anion was tested most frequently. With iodides the experimental results were not unequivocally negative. In a few experiments traces of iodine were recovered in the urine after iodides had been applied to the skin in aqueous solutions. The iodide ion has a relatively good chance to enter the skin, because, in comparison with the chloride ion, it is an anion which increases the negative electric charge of the skin and its capacity of swelling in water.[1] Also, there is some evidence that small amounts of iodides may be oxidized to free iodine in the skin and be absorbed in this form.[11]

It has been shown histologically that if there is any absorption of iodides the route is through the appendages. Whether or not this route is passed, however, may depend on different conditions in the secretion of sebum, different degree of filling of the ducts, number and size of follicles, ducts, and glands, air content, etc. The mucous layer is absolutely impermeable for iodides. The same is true for salts in general. In principle their absorption through appendages cannot be said to be impossible. Practically, however, such an absorption does not amount to more than questionable traces.

Salts of bi- and trivalent cations, especially heavy metal ions with a low electromotive force, are partly lipoid-soluble and therefore absorbable by the skin. They will be dealt with among the other lipoid-soluble substances.

Percutaneous Absorption of Water and Salts in Different Species.[1]—In lower species intake of food and output of metabolic end products are partially functions of the skin but with the phylogenetic development of the animal world the physiologic importance of absorption through the skin has gradually decreased. There is only one basic difference between percutaneous absorption in lower and higher species, however, and this concerns the absorption of water. The skin of invertebrates, fishes, and amphibians does absorb water but that of mammals does not. As for electrolytes, the tegument of even the lowest animal is impermeable if the tegument is at physiologic rest. During the period of nutrition through the skin and during the excretion of metabolic end products there is a temporary increase of permeability.

Absorption of Lipoid-soluble Substances.—The "lipoid theory" of absorption (Overton[1]) has postulated that the cell membrane consists of lipoids because only lipoid-soluble substances may penetrate the cell. Also, according to this theory the penetration of a substance quantitatively depends on the degree of its solubility in lipoids. This theory was critically reviewed in an excellent article by M. H. Jacobs.[12] Also, he amplified the theory by relating the solubility in lipoids to the nonpolar structure of organic molecules.

As far as absorption in general is concerned, many exceptions to Overton's rule were found.[12] Practically no exception could be established for absorption through the skin, however. Qualitatively it has been proved that lipoid-soluble substances penetrate the skin, whereas substances insoluble in lipoids do not. Quantitatively, however, this rule cannot be carried through consistently. Some substances slightly soluble in lipoids penetrate with greater ease than others with higher lipoid solubility. Some authors suggest that a certain degree of solubility in water together with high solubility in lipoids is optimum for absorption through the skin.[13] In other words, a certain ratio of the solubility in water and in lipoids may be the decisive factor; e.g., alcohols, aldehydes (chloral hydrate, paraldehyde), and ketones (acetone), soluble in both lipoids and water, penetrate with great ease.

The idea that the permeability to lipoid-soluble substances is due to a sterol-phosphatide framework in the cell or in the cell membrane was reviewed and experimentally supported in 1936 by Starkenstein and his co-workers.[14] These authors confirmed and supplemented earlier incomplete observations[1] which indicated that any disturbance of the lipoid framework either by precipitation or by dissolution of cholesterol breaks up the barrier of skin absorption. Previous treatment of the skin with chloroform or ether, which dissolve cholesterol, or with saponins, which precipitate it, makes the skin permeable for water-soluble substances and diminishes the permeation for lipoid-soluble compounds. On the other hand, introduction of cholesterol into the skin, for instance with ointments, diminishes the permeability for water-soluble substances because the cholesterol wall has been strengthened.[15] In fact, from a table of my earlier work[1] in which the data on the absorption of potassium iodide from different ointment bases are listed, it is obvious that the absorption is poorer from bases containing cholesterol than from bases without it.

The number of lipoid-soluble substances which have been proved to penetrate through the intact skin into the general circulation is too large to be exhaustively listed in this article. Only a few of the medically important groups of lipoid-soluble substances will be discussed, namely phenolic compounds, lipoid-soluble heavy metal salts, hormones, vitamins, alkaloids and fatty ointment bases.

The percutaneous absorption of phenol was well known to physicians of the older generation. They witnessed severe and sometimes fatal poisoning following the application of carbolic acid to large areas of the skin. Such unfortunate events did not occur frequently, however, and the popularity of this kind of treatment was not decreased for some time. Many doubted its being dangerous at all. What is more, in a very recent discussion on the permeation of phenol the question again arose as to whether its external application may cause any systemic effect.[16] The reason for this uncertainty in a problem settled a long time ago may be found in the fact that phenol in high concentration coagulates the proteins of the skin, and combines with them to form large molecules, the lipoid solubility and penetration capacity of which are completely different from those of free phenol. It should be stated here as a general rule that *any substance when applied in a high concentration having a caustic effect is absorbed in relatively smaller amounts than when the same substance is applied in lower concentrations which do not elicit a gross response.* This has been proved not only for phenol[17] but also for a number of heavy metal salts[17, 18] and for hydrogen sulfide.[19] In addition, it has been shown that previous treatment of the skin with caustics[18] or with astringents[20] decreases the permeability to other absorbable substances.

Salicylic acid (o-hydroxybenzoic acid), the most frequently tested substance in experiments on percutaneous absorption, penetrates with extreme ease through the skin from aqueous and alcoholic solutions as well as from ointments. Lipoid solubility is a property of the nondissociated molecule and not that of the salicylate ion. Sodium salicylate which is ionized in aqueous solutions is not lipoid-soluble, and does not penetrate the skin unless there is enough carbon dioxide in the tissue to liberate salicylic acid from the salt.[1, 21] The esters of salicylic acid, used externally as antirheumatic preparations, are lipoid soluble and penetrate easily.[1, 22]*

Other lipoid-soluble phenol derivatives such as resorcin, hydroquinone, pyrogallol also enter the skin from any vehicle.[16]

Of the lipoid-soluble salts of heavy metals, mercuric bichloride was often found to be absorbed from its aqueous solutions through the unbroken skin. Similarly to phenol, however, its penetration is interfered with by its coagulating effect on proteins when applied in concentrations above 0.5 per cent.[18] From the literature of past decades one may obtain the impression that systemic mercury poisoning from external application of the bichloride occurred frequently when it was used in medicated baths. One has to consider that this method of therapy was usually applied in disorders involving broken skin, however, and that with the disrupted barrier massive absorption might have occurred.

*Wintergreen oil used as a solvent increases the absorption of other lipoid-soluble substances considerably.[17]

The penetration of the skin by lipoid-soluble salts of lead such as lead acetate and lead oleate has been shown to occur from aqueous solutions.[13] The amounts absorbed are small (0.1 to 0.2 milligram daily from a 10 centimeter-square area), and it has been doubted whether they have any importance in industrial hygiene in the matter of causing clinically manifest poisoning. These traces of lead, however, as an addition to the amount absorbed by the respiratory and digestive tracts, may do their share in the development of lead poisoning.

As does lead, copper enters through the skin into the general circulation when applied in the form of its lipoid-soluble fatty acid salts, for instance copper-oxyoleic acid.[23]

Salts of lead, tin, copper, arsenic, bismuth, antimony, and mercury tend to form compounds with the fatty acid radicals of the sebum and other fats of the skin surface. Thus an originally lipoid-insoluble compound in aqueous solution may be transformed on or in the horny layer into a lipoid-soluble substance. The percutaneous absorption, then, is due to, and quantitatively dependent on, this chemical transformation. The same reaction may take place outside of the body to a larger extent if the heavy metal salt is incorporated into ointment bases which contain fatty acids. This is one reason for the increased absorption of heavy metal salts from ointments as compared with their absorption from aqueous solutions.

Because of the great therapeutic importance in dermatology of ammoniated mercury (mercuric aminochloride) its percutaneous absorption has been the object of numerous investigations. Ammoniated mercury is insoluble in water, in lipoids, and in lipoid solvents. Its bactericidal effect is based on its adsorption to the cell walls and on an extremely slow dissociation of mercuric ions under the influence of the acid reaction of the horny layer and sweat.[24] Its absorption into the general circulation is practically nil.[1, 24-27] In careful studies Gibbs and his co-workers[28] have shown that any appreciable percutaneous absorption of mercury from ammoniated mercury ointments is due to gastrointestinal absorption following contamination of the oral cavity. In adults where contamination of the mouth is much less likely to occur than in children, systemic mercury poisoning is practically never seen after the use of ammoniated mercury ointments, even if the ointment is applied daily for months or years over the whole skin surface. A few isolated cases of such poisoning which have been described[29] apparently were based on extreme hypersensitivity to mercury with traces causing poisoning.

Percutaneous absorption of vitamins and hormones strictly follows the law that any lipoid-soluble substances are absorbed through the unbroken skin in measurable amounts. Most extensive studies on absorption of lipoid-soluble hormones have been carried out with sex hormones. These studies were reviewed by Eller and Wolff in 1940.[30] It seems to make scarcely any difference quantitatively whether these hormones are given in injections or in form of ointments because the total amount is absorbed in either case. The speed of absorption is different, however, the percutaneous absorption usually being slower. The lipoid-soluble vitamins A and D and the provitamin carotene behave in the same way as do lipoid-soluble hormones.[30] The fact that irradiation of the skin with ultraviolet rays is a substitute for the intake of vitamin D in the therapy of rickets is sufficient proof of the percutaneous absorption of vitamin D.[31]

In connection with the findings on the fast permeation of lipoid-soluble vitamins and hormones the old problem once more emerged as to whether percutaneous application of drugs has any advantage over the peroral and/or parenteral ways. Such an advantage is present only if one wants to create a slowly but steadily increasing level of the drug in the blood and tissues up to a certain maximum which may then be kept constant by daily inunctions. In the past the classical mercury inunction in the treatment of syphilis was advocated mainly on this basis, although similar absorption rates and concentrations have been achieved with intramuscular injections of insoluble salts ("depot effect"). Obviously such an advantage is not sought in the case of administration of hormones and vitamins. The aim of such therapy is saturation of the deficient organism. The saturation may be achieved through the administration of the lacking factor in any mode of application, and the speed of absorption is of no great importance. If anything, rather rapid absorption is desirable in vitamin and hormonal deficiencies.

Another reason for percutaneous drug application, however, is to create a higher concentration of the drug in a certain region of the body. It has been claimed that such an effect occurs, e.g., when salicyl-ester preparations are rubbed into the skin locally above the painful muscles in myalgia. There is little doubt that introduction of a drug into the skin may temporarily cause a relatively high concentration of the drug in the tissues close to the site of application, although in reference to muscles this never has been conclusively demonstrated. When in the treatment of deep-seated organs local percutaneous therapy appears to be superior to systemic administration, this superiority results from local vasomotor reflex processes rather than from higher drug concentrations.

In the skin itself a locally higher concentration at the site of application has been unequivocally proved by the unilateral swelling[32] and unilateral hyperpigmentation[33] of the nipples in response to unilateral application of estrogenic hormone. It goes without saying that the principle of topical dermatotherapy as a whole rests on the creation of a locally high concentration of drugs in the skin without appreciable systemic effect.

In a few unduplicated experiments the lipoid-insoluble and water-soluble vitamins, ascorbic acid,[34] and thiamin chloride ("betaxin"),[35] were found to penetrate the skin. These unexpected results should not be discussed here as no confirmation could be found in the literature.

From the extensive work on percutaneous absorption of insulin[36-40] one can conclude that under carefully controlled experimental conditions negative results are prevalent. Positive results are due either to skin injuries from shaving in animal experiments[38] or to a preparatory treatment of the skin with chemicals (alkalies,[40] benzine petroleum,[41] saponins,[42, 43] irritant plant extracts[44]) which pathologically increase the permeability of the skin.

The free alkaloid bases are lipoid-soluble and penetrate the skin. Their water-soluble salts do not penetrate. For instance, nicotine, strychnine, and opium alkaloids[17] are absorbed with great ease but not their sulfate or hydrochloric salts. The impermeability for the salts of alkaloids, like that of inorganic salts, is not absolute, but only traces go through, and these only after ap-

plication for many hours. The alkaloid base in the same amount and concentration may kill the animal in a few minutes. Of anesthetics nupercaine and pantocain were claimed to have the highest capacity of penetration.[45]

It has been known since 1879 that animal and vegetable fats and oils penetrate the skin via the pilosebaceous apparatus.[1] They enter the circulating blood after having been absorbed by the sebaceous gland cells. These cells excrete microscopically visible fat droplets as their function. In viewing their structure one is not too surprised to see the reverse process also take place, namely, the intake of roughly dispersed fat by their protoplasm.

It has also been known for a long time that mineral fats and oils enter the sebaceous glands and are absorbed systemically, although their absorption is not nearly so massive as that of animal and vegetable oils and fats. All these facts have been confirmed many times, most recently by Eller and Wolff[16] in detailed and careful experiments.

Penetration of animal, vegetable, and mineral fats through the epidermis proper never has been conclusively demonstrated. Fats are not "lipoid-soluble" in the narrower sense of the term, because they form not true but colloidal-disperse solutions with fat solvents.[1] Absorption from such solutions seems to be impossible for any living cell except sebaceous gland cells. Lanolin and paraffin are not absorbed by the epithelial cells of the intestines either,[1] and triglyceride fats are absorbed along this route only after they have been split into fatty acids and glycerin.

Absorption of Water-Soluble, Lipoid-Insoluble Nonelectrolytes.—Theoretically such absorption is not to be expected through a membrane which is impermeable to water. Experimentally it has been shown that sucrose[46] and galactose[47] do not penetrate from ointments, and it is highly probable that glucose does not either. It is regrettable that the available data on this problem are deficient and unsatisfactory.

Immunologic and allergic experiments seem to indicate that lipoid-insoluble antigenic substances which usually are large molecules or molecule aggregates may enter the skin from ointments.[48] This unexpected result, however, has been achieved only after rubbing the antigen-ointment vigorously into the skin for fifteen minutes. It has been stated that such treatment irritates the skin. A skin area treated in this way cannot be regarded as normal. It must also be considered that in the sensitized organism traces of the antigen are sufficient to elicit the immunologic reactions which have been used as indicators for the absorption of the antigens. That their absorption is not great in amount is indicated by recommendations to increase it either by previous treatment of the skin with benzene and petroleum benzine,[49] or by electrophoretic introduction,[50, 51] or by the use of the newly described penetrasols of Herrmann, Sulzberger, and Baer.[69]

Absorption of Gases.—Substances which are in the gaseous form at room temperature easily penetrate the skin with the remarkable exception of carbon monoxide which is insoluble in lipoids.[1] Ease of percutaneous absorption has been demonstrated for the following gases: oxygen,[1, 52] nitrogen,[53] helium,[53] carbon dioxide,[1, 10] ammonia vapor, hydrogen sulfide,[1, 10] hydrogen cyanide,

vapors of nitrobenzene, dinitrobenzene, dinitrotoluene,[1] and vapors of volatile aromatic oils.[17]

When hydrogen sulfide is applied to the skin in irritating concentrations, its absorption is relatively smaller than in low concentrations.[19] Experiments with aqueous solutions of hydrogen sulfide at various pH's[54] have revealed that the nondissociated H_2S molecule penetrates better than do the HS^- or NaS^- ions. Ammonium sulfide, however, seems to penetrate better than hydrogen sulfide.[55] If elemental sulfur is applied to the skin in ointment vehicles, its absorption occurs in the form of sulfides.[56] Cases of poisoning after treatment with sulfur ointments have been reported as due to this mechanism.[57] The manifestations are those of hydrogen sulfide poisoning.

The absorption rate of oxygen and carbon dioxide at constant temperature and pressure depends exclusively on the difference in the concentration of these gases within and outside of the skin.[1, 52] This absorption thus represents a simple physical diffusion process. This is probably true also for gases foreign to the body. The finer mechanism of this diffusion process has not been studied in detail. Its importance for toxicology and industrial hygiene is evident.

The Role of Vehicles.—Substances soluble in both water and lipoids such as salicylic acid or mercuric bichloride penetrate with great ease from aqueous solutions as well as from ointments or from fat solvents. Although usually the absorption from fats and fat solvents is more rapid and more complete than from water, this is not a general rule. It has been found, for instance, that estrogenic and androgenic hormones are better absorbed from aqueous vehicles than from oil.[30, 58] Such findings indicate that the permeation of a substance depends primarily upon its own (total or partial) lipoid solubility. The kind of vehicle into which it is incorporated and the solubility of the substance in the vehicle are of secondary importance.

The use of fat solvents, such as ether, chloroform, benzene, petrol-benzine, and, to a lesser degree, alcohol, usually enhances the percutaneous absorption considerably. This action is due not to the high solubility of lipoid-soluble substances in these solvents, however, but to the fact that these solvents in themselves increase the permeability of the skin. If the skin is treated with them in advance, i.e., previous to the absorption experiment, an increased absorption is subsequently observed from aqueous solutions as well as from any other vehicle. The classical experiments of Winternitz[1] with aqueous solutions of strychnine salts have proved this fact, and more recent observations have confirmed his results.[14]

Organic solvents may accelerate absorption by dissolving the greasy cover of the skin surface and of the follicular wall, thereby facilitating the wetting of these surfaces by aqueous solutions. This action is a minor factor, however. The main action of these solvents lies in their dissolving of lipoid compounds in the living cells themselves, thus disrupting the barrier of absorption.

Ointments.[1]—Ointments are fats or similar substances of a greasy consistency. They are applied to the skin with or without incorporated drugs, mainly for therapeutic purposes. Ointments can be spread over the skin in a continuous thin layer. This cannot be done with aqueous or alcoholic solutions, because their surface tension working against a low viscosity causes

diminution of the surface, and formation of drops with disruption of the layer results.*

In order to bring the thinly spread ointment layer close to the uneven surface of the skin, a pressure must be exerted. By the pressure the gas bubbles adherent to the pores escape sideways; deeper in the follicles and gland ducts, they become compressed and absorbed. Applying the same pressure, one will obtain a closer contact with the surface if the viscosity is low, i.e., if the ointment is flexible and supple. Ointments which have a high viscosity and cannot be spread in thin layers are called tenacious or tough.

Numerous data have been presented indicating that incorporation of a substance into an ointment increases its rate of absorption in comparison with the absorption from other vehicles. Such an effect is mainly due to the mechanical factors as described. One presses the ointment into the follicle, and thereby brings the substances more rapidly and in larger amounts to the absorbing surface of the sebaceous gland cells than is possible with other vehicles. The ointment bases are absorbed through the appendages exclusively, and therefore they can increase the absorption of incorporated substances only via this route. The importance of the mechanical factor is indicated by the fact that absorption is furthered more readily by supple ointments than by tenacious ones.

Although ointments are helpful in bringing the incorporated substance closer to the surface of absorbing cells, *there is no evidence that either ointments or any other kind of "vehicle" may serve as a transportation vehicle into the cell itself.*[14] If we choose a substance to which the skin is completely impermeable, e.g., sodium salicylate, we are unable to enforce absorption even with the most supple ointment and despite extreme pressure.[1] Data are available indicating that vehicle and incorporated substance are absorbed separately and at different rates of speed. It was found, for instance, that from the metallic mercury ointment (blue ointment) at first more of the ointment base and later more of the mercury was absorbed.[59] In addition, some experimental results indicate that the incorporated substance has to separate from the ointment base before it enters the cell; the greater the tendency to separation, the more intensive is the absorption.[60]

The problem of determining the composition of an ointment which would attain the maximum absorption of incorporated substances has received much attention. With the exception of the requirement of suppleness no general rule could be established. It has been shown that for various substances different ointment bases are optimal.[56] In general, however, the addition of cholesterol as an emulsifier does not favor absorption.[15]

Considering the importance of low surface tension and of thorough wetting of the follicular wall it was to be expected that application of the modern wetting agents (synthetic detergents) would enhance percutaneous absorption considerably. This was demonstrated recently by W. Duemling.[61]

Chemical reactions between substances and ointment vehicles may increase absorption, if by such reactions lipoid-insoluble substances are transformed into

*There are two surface tensions to be dealt with: one between skin and liquid, and the other between liquid and air. Both act in the same direction if the liquid does not wet the skin, as is the case with water. With alcohol the two tensions act in opposite directions; nevertheless, formation of drops occurs.[1]

soluble ones. The most common chemical reaction is the formation of fatty acid salts (soaps) from neutral salts.

Electrophoresis.[1]—By applying a galvanic current to the skin, substances can be introduced into the circulation which otherwise would not penetrate or would do so only in traces. "Electrophoresis," meaning electric transfer, is the most suitable term for this procedure because it does not involve any assumption concerning its mechanism. The term "cataphoresis" is properly reserved for the transport of colloidal particles in an electric field. The expression "ion transfer" or "iontophoresis" involves the assumption that only ions are transferred in one direction or the other depending on their charge. The term excludes the transfer of nondissociated molecules. "Electroendosomosis" is defined as the movement of a liquid through an electrically charged membrane under the influence of a galvanic current.

Attempts to utilize electrophoresis in clinical therapy can be traced back to the middle of the last century. Again and again it was rediscovered and was believed to accomplish more than other methods of application. In spite of some interesting results, such as histamine electrophoresis in myalgia and mecholyl iontophoresis in arthropathia, the therapeutic achievements of electrophoresis have been rather meager up to date. Possibly, more recent efforts to utilize this method in diagnostic procedures[50, 51] will be more successful.

The method of clinical electrophoresis is simple. The two poles of the galvanic current are connected with aluminum or zinc plates which are covered with gauze. The "neutral" electrode is soaked with some inactive electrolyte solution, usually tap water. It is firmly applied anywhere on normal skin, or it is clipped to the inside of an insulated vessel (enamel bowl) filled with tap water into which the patient's limb is immersed. The "active" electrode has a relatively small surface, thus producing a high electrical density, i.e., high milliamperage per square unit area. After its gauze has been soaked with the solution to be introduced, the active electrode is pressed and firmly fixed to the skin area to be treated, or anywhere on the skin surface if systemic effects are sought. A current of varying intensity from a few tenths of a milliampere up to 40 milliamperes (in the average 1 milliampere per 1 cm. square area) is then sent through the body for a period of time varying from a few minutes up to one-half hour.

When substances are introduced into the skin electrophoretically, both ion transfer and electroendosomosis are involved. The mechanism of ion-transfer is well understood. If the biologically active part of an electrolyte is a cation, the drug is applied to the positive pole (the anode), and the cation will travel toward the negative pole, i.e., towards the inside of the body. The inactive anion will travel in the opposite direction, i.e., from the soaked gauze to the metal electrode. Similarly, a substance with an active anion will penetrate to the inside of the body from the negative pole (cathode). Thus iodide and bromide ions are easily introduced from the negative pole, metal ions and alkaloid-cations from the positive pole.

Electroendosmosis, the transport of the liquid as a whole, however, interferes with this simple mechanism.[2, 3, 62] The skin has a negative electric charge, and therefore electrophoresis will effect a movement of water into the body from the positive pole, and toward the outer surface of the skin at the negative pole.

A gross clinical sign of this movement of water is a shrinking of the skin pores at the positive, and a swelling at the negative pole after intensive electrophoresis. In case of cation-transfer from the positive pole, electroendosmosis acts in the same direction, thus facilitating the absorption of the cation. The anion-transfer from the negative pole, however, is counteracted by endosomosis, which elicits a movement in the opposite direction.

Difficulties encountered in the introduction of dye anions ("acid dyes") were interpreted as being due to the lack of endosmosis in the same direction.[62]

The role of endosmosis was proved by experiments indicating that any factor which furthers electroendosmosis also favors electrophoretic introduction from the positive pole.[3, 62, 63] Such factors are the addition of sucrose or of alcohol to the aqueous solutions of drugs or dye stuffs containing an active cation, factors which are unfavorable for ion-transfer but enhance electroendosmosis. On the other hand. high salt concentration or acidification has an opposite effect; they are unfavorable for electroendosmosis and greatly hinder permeation through the skin.

In contrast to cation-introduction, the entrance of anions is furthered if electroendosmosis is suppressed by the addition of acids, of multivalent cations or by concentrated salt solutions. The addition of sucrose or alcohol inhibits the entrance of anions.

The effect of electrophoresis on percutaneous absorption has been studied mainly with respect to aqueous solutions of dyes and alkaloid salts.[1] A great many of the latter, such as salts of strychnine, morphine, and atropine which have no demonstrable effect when painted on the skin in aqueous solution, display their characteristic general toxic effects after a short time when they are introduced electrophoretically in the same amounts and concentrations. The electrophoretic introduction of other drugs may be demonstrated by local effects at the site of entrance. Local vasoconstriction by epinephrine salts, local sweating by salts of acetylcholine, mecholyl, and pilocarpine, and local anesthesia by salts of cocaine cannot be produced by simple application of their aqueous solutions to the skin but are easily achieved by introduction with the galvanic current. The decided effect of electrophoresis on skin absorption was demonstrated dramatically by Leduc in 1900.[1] A rabbit painted with a solution of strychnine nitrate survived this treatment without the slightest signs of poisoning, whereas another rabbit into which the same solution was introduced electrophoretically died in a few minutes from strychnine poisoning.

In spite of this remarkable effect of electrophoresis one has to keep in mind that application of galvanic current does not alter the essential principles governing skin absorptivity. Aqueous solutions of electrolytes enter the skin in traces through the appendages without the use of any current. If an aqueous solution of strychnine nitrate in high concentration is painted on the skin over large cutaneous surfaces and for a long period of time, it will pass the appendages in amounts which eventually will kill the animal.[1] The electric current serves only to increase this absorption through the appendages, but it does increase it tremendously. Under the conditions of medical electrophoresis, the current carries aqueous solutions through the appendages exclusively and not through the epidermis. With respect to dyes this principle was demonstrated as early as 1890,[1] and has often been confirmed since.[64, 65]

Under conditions favorable for electroendosmosis in the electrophoretic experiment, an aqueous solution may enter the Malpighian layer, but then, as shown in experiments with dyes, the solution penetrates through the follicular wall and not through the surface epithelium.[62] In other words, the barrier in the follicular epithelium which has a histologic and physicochemical structure similar to that of the surface epithelium, offers less resistance to the current than does the epidermal barrier. The latter forms the greater obstacle.

The electric current itself also travels through the appendages. If applied to the skin with electrodes which are soaked with saline solution previous to the absorption experiment, the current will increase the absorption of aqueous solutions which are subsequently painted on the skin. This effect is due to decrease of polarization. Decrease of polarization, however, is a minor factor if galvanic; i.e., noninterrupted direct current is applied.[1] The main effect of electrophoresis is the great increase of absorption through the appendages without qualitative change of the permeability of the skin.

Absorption Through Diseased Skin.[1]—The study of absorption through pathologically changed skin corroborates the basic rules of percutaneous absorption: (1) The thickness of the horny layer is of no importance (normal absorption occurs from ichthyotic skin); (2) the fatty cover of the skin is irrelevant (there is equal absorption from seborrheic and dry skin); (3) the barrier is in the epidermis (destructive lesions of the epidermis eliminate the physiologic barrier); (4) the most important way of absorption is through the appendages (decreased absorption occurs in scleroderma and other processes with associated atrophy of the appendages).

Recent Contributions.—Miescher[65] studied the penetration of water-soluble fluorescent dyes by applying them in different vehicles to the skin of laboratory animals. The depth of cutaneous penetration was noted by observing frozen sections under the fluorescence microscope. He found only diffusion into the upper loose horny layer, and thus corroborated the existence of a barrier at the lower level of the stratum corneum. Furthermore, he confirmed the observations indicating that the thickness of the horny layer is of no influence on the absorption through the skin.

The increasing use and extreme efficiency of topical application of sulfonamides in war surgery justify the interest in their percutaneous absorption. Recent experimental work[66] has shown that sulfanilamide penetrates the intact skin from organic solutions. Sodium and calcium salts of sulfathiazole enter the skin from aqueous solutions and from ointments; they also can be introduced electrophoretically.[67] On the basis of what has been said in this review, the lipoid-soluble sulfonamides may be expected to penetrate the skin from organic solvents and ointments. Aqueous solutions of the sodium salts should be applied to the intact skin only by electrophoresis. One cannot expect, however, that the electrophoresis of the sulfanilamide anion from the negative pole will be sufficiently effective, because of the antagonistic effect of the opposite direction of the forces of electroendosmosis.

Herrmann, Sulzberger, and Baer[68] have recently introduced new types of vehicles. These vehicles were designed to increase percutaneous absorption by combining the properties of fat or lipoid solubility, water solubility, surface-

wetting action, and actions as solvents and homogenizers. An example of such a combined vehicle and solvent consists of xylene (or derivatives), a synthetic wetting agent, antipyrine (for solution and homogenization), and propylene glycol. In their preliminary report, the authors have called vehicles of this type "penetrasols."

The authors state that these pentrasols accelerate the penetration of a great variety of substances through grossly intact human and animal skins (sulfonamides, heavy metals, so-called protein allergens, etc.). The reviewer is of the opinion that the effects reported exceed those to be expected by simple lowering of the surface tension and by thorough wetting of the surface (see. p. 19). The penetrasols developed by Sulzberger, Herrmann, and Baer may achieve their effects by attacking the lipoid frame of the living epidermal cells in a manner similar to the effect exercised by saponins.

REFERENCES

1. Rothman, S.: Resorption durch die Haut. Handbuch der normalen und pathologischen Physiologie 4: 107, 1929, and Supplements 18: 85, 1932.
2. Rein, H.: Die Gleichstrom-Leiter-Eigenschaften und elektromotorischen Kräfte der menschlichen Haut und ihre Auswertung zur Untersuchung von Funktionszuständen des Organes, Ztschr. f. Biol. 85: 195, 1927.
3. Rein, H.: Experimentelle Studien über Elektroendosmose an überlebender menschlicher Haut. Ztschr. f. Biol. 81: 125, 1924.
4. Ebbecke, U.: Die lokale galvanische Reaktion der Haut. Arch. f. d. ges. Physiol. 190: 230, 1921.
5. Rothman, S.: Biochemie der Haut und Hautanhänge. Oppenheimer's Handbuch der Biochemie des Menschen und der Tiere. 2nd Edition. Suppl. Vol. 2: 157, 1934.
6. Hediger, S.: Experimentelle Untersuchungen über de Resorption der Kohlensäure durch die Haut. Klin. Wchnschr. 7: 1553, 1928.
7. Bürgi, E.: Über die Möglichkeiten und Grenzen der perkutanen Therapie. Schweiz. med. Wchnschr. 18: 433, 1937.
8. Gray, J.: Experimental Cytology. Cambridge, 1931, p. 349-407.
9. Kahlenberg, L.: On the Passage of Boric Acid Through the Skin by Osmosis. J. Biol. Chem. 62: 149, 1924.
10. Lehmann, G.: Über die Resorption von Kohlensäure aus Salzlösungen und von Salzlösungen selbst durch die Haut. Arch. internat. de pharmacodyn. et de thérap. 55: 331, 1937.
11. Günther, G.: Experimentelle Studien über die Hautresorption, Prag. Arch. f. Tiermed. u. vergleich. Path. 6: 55, 1926.
12. Jacobs, M. H.: Permeability of the Cell to Diffusing Substances, Section III of Cowdry's General Cytology, 1924, p. 99.
13. Süssmann, P. O.: Studien über die Resorption von Blei und Quecksilber bzw. deren Salzen durch die unverletzte Haut des Warmblüters. Arch. f. Hyg. 90: 175, 1922.
14. Starkenstein, E., and Hendrych, F.: Zur Physiologie und Pharmakologie der Sterine. II. Die Bedeutung des Cholesterins für Permeabilität und Resorption. Arch. f. exper. Path. u. Pharmakol. 182: 664, 1936.
15. Matschak, H.: Zur Physiologie und Pharmakologie der Sterine. III. Der Einfluss der Sterine in Salbengrundlagen auf die Resorption percutan verabreichter Arzneimittel, Arch. f. exper. Path. u. Pharmakol. 182: 688, 1936.
16. Eller, J. J., and Wolff, S.: Permeability and Absorptivity of the Skin. Arch. Dermat. & Syph. 40: 900, 1939.
17. Macht, D. I.: The Absorption of Drugs and Poisons Through the Skin and Mucous Membranes, Arch. internat. de pharmacodyn. et de thérap. 58: 1, 1938.
18. Bürgi, E.: La perméabilité de la peau au mercure, Rev. méd. de la Suisse Romaine 57: 461, 1927.
19. Schmid, G.: Über die Resorption von Kohlensäure und Schwefelwasserstoff durch die Haut. Arch. internat. de pharmacodyn. et de thérap. 55: 318, 1937.
20. Meyenberg: Über das Einverleibungsvermögen (Resorptionsvermögen) der Haut. Dermat. Wchnschr. 112: 31, 1941.
21. Hopmann, F.: Über den Einfluss des Kohlensäurebades auf die Permeabilität der Haut für Salicylsäure. Der Balneologe 6: 5, 1939.
22. Brown, E. W., and Scott, W. O.: The Absorption of Methyl Salicylate by the Human Skin. J. Pharmacol. & Exper. Therap. 50: 32, 373, 1934.
23. Schmid, R., and Winkler, J.: Über die kutane Kupferresorption aus einer Kupfer enhaltenden Salbe. Klin. Wchnschr. 17: 559, 1938.

24. Perutz, A.: Die Pharmakologie der Haut, Jadassohn's Handbuch der Haut- und Geschlechtskrankheiten 5/1: 2, 1930.
25. Current Comment, J. A. M. A. 107: 1722, 1936.
26. Maloff, G.: Über die Resorption des Quecksilbers nach Applikation der weissen Präzipitatsalbe auf die Haut. Deutsche med. Wchnschr. 51: 1381, 1928.
27. Moncorps, C.: Untersuchungen über die Pharmakologie und Pharmakodynamik der Salben und salbeninkorporierten Medikamente. VI. Über die Pharmakologie und Pharmakodynamik des Ungt. Hydr. praec. alb. Ph. G. Arch. f. exper. Path. u. Pharmakol. 155: 51, 1930.
28. Gibbs, O. S., Shank, R., Pond, H., and Hansmann, G. H.: Absorption of Externally Applied Ammoniated Mercury, Arch. Dermat. u. Syph. 44: 862, 1941.
29. Alexander, A., und Mendel, K.: Chronische Quecksilbervergiftung durch langdauernden Gebrauch einer Sommersprossensalbe, Deutsche med. Wchnschr. 49: 1021, 1022, 1923.
30. Eller, J. J., and Wolff, S.: Hormones and Vitamins in Cosmetics, J. A. M. A. 114: 1865, 2002, 1940.
31. Sulzberger, M. B.: Discussion of 16.
32. Jadassohn, W., Uehlinger, E., and Margot, A.: The Nipple Test. Studies in the Local and Systemic Effects on Topical Application of Various Sex Hormones, J. Investig. Dermat. 1: 31, 1938.
33. Davis, M. E., Boynton, M. W., Ferguson, S., and Rothman, S.: Studies on Estrogenic Pigmentation, Jour. Clin. Endocrinol. In press.
34. Kasahara, M., und Kawashima, K.: Die Resorption des Vitamin C durch die Haut, Klin. Wchnschr. 16: 135, 1937.
35. Kasahara, M., Hayashi, J., Yokonowa, T., und Furumi, R.: Die Resorption des Vitamin B durch die Haut, Klin. Wchnschr. 17: 939, 1938.
36. Telfer, S. V.: The Administration of Insulin by Inunction, Brit. M. J. 1: 715, 1923.
37. Hermann, S., und Kassowitz, H.: Experimentelle Grundlagen der perkutanen Insulinwirkung, Klin. Wchnschr. 14: 1531, 1933.
38. Bruger, M., and Flexner, J.: Integrity of the Skin in Relation to Cutaneous Absorption of Insulin, Proc. Soc. Exper. Biol. & Med. 35: 429, 1936.
39. Maier-Weinertsgrün, D.: Versuche mit der Insulinsalbe Hocutan, Klin. Wchnschr. 15: 1245, 1936.
40. Major, R. H., and Delp, M.: Cutaneous Absorption of Insulin, Proc. Soc. Exper. Biol. and Med. 37: 338, 1937.
41. Tanigawa, K.: On the Percutaneous Absorption of Insulin, Mitt. a. d. med. Akad. zu Kioto 30: 273, 1940.
42. Kingisepp, G., and Talli, L.: Percutane Insulinresorption, Klin. Wchnschr. 18: 1323, 1939.
43. Milbradt, W.: Der Einfluss der Saponine auf die Durchgängigkeit der Haut, Ztschr. f. d. ges. exper. Med. 84: 745, 1933.
44. Abren, B. E., and Emerson, G.: Absorption of Insulin With Mucuna Pruriens, Univ. California Publ., Pharmacol. No. 3. 1: 49, 1938.
45. Freystadtl, B.: Experimenteller Nachweis der Penetrationsfähigkeit einiger Oberflächenanästhetika durch die unverschrte Haut, Dermat. Wchnschr. 106: 73-79, 1938.
46. Winternitz, H., and Naumann, H.: Gibt es eine Ernährung durch die Haut? Deutsche med. Wchnschr. 55: 1828, 1929, und 56: 492, 1930.
47. Bauke, E. E.: Wird Milchzucker von der Haut resorbiert? Deutsche med. Wchnschr. 56: 1869, 1930.
48. Walzer, A.: Cutaneous Absorption. I. A Direct Technic for Demonstrating Antigens, Arch. Dermat. & Syph. 41: 692, 1940.
49. Ishikawa, R.: Immunological Studies on the Function of the Skin. Part III. On the Percutaneous Immuno-Therapy, Mitt. a. d. med. Akad. zu Kioto 25: 900, 1939.
50. Abramson, H. A.: Skin Reactions. X. Preseasonal Treatment of Hay Fever by Electrophoresis of Ragweed Pollen Extracts Into the Skin, J. Allergy 12: 169, 1941.
51. Shilkret, H. H.: Electrophoretic Skin Studies. I. Reaction to Common Grasses, J. Investig. Dermat. 5: 11, 1942.
52. Shaw, L. A., and Messer, A. C.: Cutaneous Respiration in Man. III. The Permeability of the Skin to Carbon Dioxide and Oxygen as Affected by Altering Their Tension in the Air Surrounding the Skin, Am. J. Physiol. 98: 93, 1931.
53. Behnke, A. R., and Willmon, F. L.: Cutaneous Diffusion of Helium in Relation to Peripheral Blood Flow and the Absorption of Atmospheric Nitrogen Through the Skin, Am. J. Physiol. 131: 627, 1940-1941.
54. Roques, E., Caujolle, F., and Roy, J.: La permeabilité cutanée à l'hyposulfite de sodium, à l'hydrogène sulfuré et aux sulfures de sodium, Bull. Acad. de méd. Paris 119: 515, 1938.
55. Draize, J. H., and Laug, E. P.: The Percutaneous Absorption of Ammonium Hydrogen Sulfide and Hydrogen Sulfide, J. Pharmacol. & Exper. Therap. 76: 179, 1942.
56. Moncorps, C.: Über die Pharmakologie und Pharmakodynamik von Salben und salbeninkorporierten Medikamenten, Arch. f. exper. Path. u. Pharmakol. 141: 50, 1929, and 152: 57, 1930.

57. Basch, F.: Über Schwefelwasserstoffvergiftung bei äusserlicher Applikation von elementarem Schwefel in Salbenform, Arch. f. exper. Path. u. Pharmakol. 111: 126, 1926.
58. Nelson, D., Greene, R. R., and Wells, J. A.: Variations in the Effectiveness of Percutaneously Applied Androgens in the Rat, Endocrinology 26: 651, 1940.
59. Wild, R. B., and Roberts, J.: The Absorption of Mercurials From Ointments Applied to the Skin, Brit. M. J. 1: 1076, 1926.
60. Unna, P., Jr.: Zur Frage der Salbengrundlagen, Deutsche med. Wchnschr. 52: 197, 1926.
61. Duemling, W. W.: Wetting Agents. New Synthetic Chemicals of Use in Finer and More Efficient Topical Dermatologic Therapy, Arch. Dermat. & Syph. 43: 264, 1941.
62. Rein, H.: Zur Elektrophysiologie der menschlichen Haut, Ztschr. f. Biol. 84: 41, 1926.
63. Raszeja, F.: Recherches experimentales sur l'ionothérapie electrique. Etudes histochimiques et biologiques, Compt. rend. Soc. de biol. 103: 799, 1930.
64. Abramson, H. A., and Gorin, M. H.: Skin Reaction. IX. The Electrophoretic Demonstration of the Patent Pores of the Living Human Skin; Its Relation to the Charge of the Skin, J. Physiol. Chem. 44: 1094, 1940.
65. Abramson, H. A., and Engel, M. G.: Skin Reactions. XII. Patterns Produced in Skin by Electrophoresis of Dyes, Arch. Dermat. & Syph. 44: 190, 1942.
66. Zondek, B., Bromberg, Y. M., and Shapiro, B.: Percutaneous Application of Sulfanilamide in Animals and Men, Proc. Soc. Exper. Biol. & Med. 50: 116, 1942.
67. Clark, W. G., Strakosch, E. A., and Nordlum, C.: Penetration of Sulfonamides Through Intact Skin by Iontophoresis and Other Means of Local Application, Proc. Soc. Exper. Biol. & Med. 50: 43, 1942.
68. Miescher, G.: Fluoreszenzmikroskopische Untersuchungen zur Frage der Penetration von fluoreszierenden Stoffen in die Haut, Dermatologica 83: 50, 1941.
69. Herrmann, F., Sulzberger, M. B., and Baer, R. L.: New Penetrating Vehicles and Solvents, Science 96: 451, 1942.

A SIMPLE LIVER PREPARATION ORALLY EFFECTIVE IN THE TREATMENT OF HUMAN AMYLOID DISEASE*

JAMES LOUKIDES, B.SC., HAROLD G. GRAYZEL, M.D., AND
MENDEL JACOBI, M.D., F.A.C.P., BROOKLYN, N. Y.

ORALLY administered powdered whole liver can effect the resorption of experimentally produced amyloid in animals,[1] and can effect an apparent clinical cure in human beings with secondary generalized amyloidosis.[2] In the latter paper it was shown that the administration of such a liver preparation caused the amyloid to disappear, despite the continuing activity of the incitant inflammatory disease.

Many requests for the liver product or its method of preparation have come to us from physicians all over the country. The liver powder used in our original work is no longer available, the concern producing it having ceased operation. We have tried the leading commercial liver preparations, but have found them unsatisfactory in the treatment of secondary amyloidosis. We therefore believe that a brief description of a method of preparing a "liver cocktail," with which we have had results as good as those with the powdered liver originally used by us and which is inexpensive and easy to prepare, is in order.

METHOD OF PREPARATION

Liver obtained fresh from an abattoir is cleansed of its gross extraneous matter, especially gross blood vessels. It is then cut into cubes and passed

*From Neponsit Beach Hospital for Children, Jewish Hospital of Brooklyn, and Beth-El Hospital, Brooklyn, N. Y.

Received for publication, November 27, 1942.

through a Sep-Ro-Siv.* The very fine sieve of the latter is used, the liver forced through the machine several times in order to reduce the product to a fine, pasty consistency.

Fruit juices are added to wash as much of the liver as possible through the sieve of the machine. The finely mashed liver will protrude through the pores of the sieve, while any attached tough muscle, fibrous tissue, or large blood vessels will be extruded through the end of the funnel. This residue from the funnel should again be put through the machine several times, for it thereby helps clean the teeth of the grinder of considerable retained valuable liver mash. A spoon is finally used to free the sieve of adherent macerated liver.

The pressed tissue and the juice are collected in a bowl. Additional fruit juice is added to thin out the mixture, and this is then passed through a fine scoop sieve that can be purchased in any five-and-ten-cent or hardware store. The finished mash should be totally free from lumps of liver. It generally resembles a chocolate drink in color. The taste is that of the fruit juice used, is quite palatable, and is taken readily. A mixture of orange and pineapple juice is the vehicle most popular with our patients; any juice, however, may be used to suit the patient. The liver preparation should be kept in a cold refrigerator or icebox. In the event that the drink is too thick, additional fruit juice may be added to suit the patient's demand. At certain times of the year the liver may have a pungent taste. Washing one's mouth with unadulterated fruit juice, or chewing a few gum drops will easily and rapidly overcome this taste.

This procedure gives an approximate yield of 90 per cent. The dosage varies from the equivalent of ¼ to ¾ of a pound of liver daily. Wherever possible, the use of mash prepared daily is recommended. We have found, however, that a three-day supply kept at a temperature of 8° F. or lower is equally potent and palatable. After four or five days the juice becomes mildly sour. Two pounds of liver and one quart of fruit juice yield a total of about two quarts of the final mash, a supply sufficient for three days.

COMMENT

We have found this fresh whole liver preparation effective against amyloid. It has the advantage of being simple to prepare and of being far less expensive than any commercial product. It has the disadvantage of requiring a rather tedious and time-consuming procedure, and of daily preparation or, at most, at intervals of three days. Conscientiously adhered to, the yield in quantity and potency of the product is high, and the drink is pleasant in appearance and taste.

There should be no possibility of ingesting parasites in the liver if the material is obtained from a reliable slaughterhouse and the liver is carefully examined as it is cubed. We have found no parasites in any of our liver preparations after prolonged and careful search, including microscopic examination of the product.

REFERENCES

1. Grayzel, H. G., Jacobi, M., Warshall, H. B., Bogin, M., and Bolker, H.: Amyloidosis, Arch. Path. 17: 50, 1934.
2. Grayzel, H. G., and Jacobi, M.: Secondary Amyloidosis: Results of Therapy With Desiccated Whole Liver Powder, Ann. Int. Med. 12: 39, 1938.

*Sep-Ro-Siv is manufactured by the Chisholm Ryder Co., Niagara Falls, N. Y., and is distributed through the American Utensil Co., 460 West Superior St., Chicago, Ill.

THE PROPHYLACTIC USE OF SULFANILAMIDE IN CHILDREN WITH RHEUMATIC HEART DISEASE*

CHARLES R. MESSELOFF, M.D., AND MILTON H. ROBBINS, M.D.,
NEW YORK, N. Y.

IN JANUARY, 1939, Coburn and Moore,[1] as well as Thomas and France,[2] published independently their observations on the prophylactic effect of sulfanilamide in rheumatic children. Stimulated by their favorable results, we began, in September, 1939, to administer sulfanilamide to a group of children attending our cardiac clinic. This communication is a report of the result of our study.

SELECTION OF CASES AND PROCEDURE

Observations were begun on children with inactive rheumatic heart disease who were attending the clinic. As the study progressed, a number of children were eliminated from the treated group and transferred to the control group because of (1) the appearance of mild toxic effects of sulfanilamide, or (2) lack of cooperation by either the child or the parent.

In all, 55 children were observed. There were 25 children in the treated group, and 30 in the control group. They varied in age from 6 to 14 years at the time of the beginning of the study. A study of Table I will show that, using the age of 12 years as the beginning of puberty, both groups consisted of almost equal numbers of pre- and postpuberal children.

TABLE I

AGE DISTRIBUTION AND SEX OF TREATED AND CONTROL PATIENTS

AGE	TREATED GROUP	CONTROL GROUP
6-8	1	3
9-11	12	9
12-14	12	18
Total	25	30

	TREATED GROUP		CONTROL GROUP	
	BOYS	GIRLS	BOYS	GIRLS
Number:	12	13	17	13

From the outset, we followed one method of medication: 5 grains (0.3 Gm.) of sulfanilamide were given 3 times daily for a period of one week, and if no toxic signs or symptoms appeared, the dose was increased to 10 grains (0.6 Gm.) in the morning and in the evening. This dose was maintained for the remainder of the study.

*From the Cardiac Service of the Hospital for Joint Diseases.
Received for publication, January 29, 1942.

The drug was administered during two treatment seasons: (1) from September, 1939, through May, 1940, and (2) from September, 1940, through May, 1941. The actual period of treatment during each season was about eight months.

A complete blood count, sedimentation rate, and an electrocardiogram were obtained on each child prior to the initial administration of the drug. Throughout the period of observation, blood counts and sedimentation rates were obtained at two- or three-week intervals. At similar intervals, a careful history and examination were done to determine the presence either of ill effects of sulfanilamide or of evidence of rheumatic activity. The children in the control group were seen, on the average, every three months unless a rheumatic recrudescence occurred. In such instances, the children were seen more frequently.

TOXIC EFFECTS OF SULFANILAMIDE

We can corroborate the observations of Thomas and her co-workers[3] that the serious toxic signs and symptoms which are not infrequently noted following the administration of large doses of sulfanilamide are not seen when small daily doses are taken. Thus, cyanosis, severe hemolytic anemia or granulocytopenia, severe dermatitis, febrile reactions, were not seen in our series. Minor toxic effects, however, were observed in ten children. Recovery was prompt and complete in every child following cessation of therapy. Indeed, experience has shown that recovery may take place in some children even if the drug is continued. We did not deem it justifiable, however, to expose ambulatory children to the continued effect of sulfanilamide in the presence of even minor drug reactions.

It is of interest to record, briefly, the toxic effects observed in our group.

1. *Anorexia.*—V. A., a 14-year-old Italian boy, stated that he lost his appetite a few days after he began taking sulfanilamide. The drug was discontinued and was resumed three months later. Again his appetite diminished, and he lost weight. Sulfanilamide was stopped.

2. *Anemia.*—A moderate decrease in the hemoglobin content was found in four cases, accompanied in only one instance by a drop in the red blood cell count. In the latter case, the hemoglobin value fell from 12 Gm. to 8.6 Gm., and the red blood cell count from 4 million to 3,200,000. Upon stopping the drug these values rose to their premedication level. Three months later, the same child was again given sulfanilamide, but this time there was no change in either the hemoglobin or the red blood cell value.

3. *Leucopenia.*—In six children the total white blood count dropped below 5,000, but in only two of these children did granulocytopenia (mild in degree) accompany the leucopenia. Therapy was continued in the four children who showed a leucopenia alone, and in all, the leucocyte count rose to normal levels. The leucopenia observed in these children was mild: between 4,000 and 5,000 in three children, and 3,700 in the fourth child.

Therapy was discontinued in the two children who showed leucopenia and granulocytopenia. In both cases the blood counts returned promptly to normal.

4. *Rash.*—A mild maculopapular eruption was observed on the arms and legs of one child, and on the face of another. Both eruptions disappeared soon after stopping the drug.

5. *Allergic Reaction.*—Finally, one child, a 13-year-old Italian girl, complained of a severe frontal headache, which came on three hours after the initial dose of sulfanilamide and which persisted for five days until she discontinued taking the drug. One month later, she was again given sulfanilamide, but this time she did not complain of headaches. After a week of medication, however, "swelling of both knees, elbows, and shoulders" appeared during one night, only to disappear the next morning. She continued taking the drug. Two days later, her face became swollen and remained so for forty-eight hours. Sulfanilamide was discontinued.

RESULTS OF TREATMENT

Evidence of rheumatic reactivation appeared in both the treated and the control groups. In the treated group of twenty-five children, three manifested rheumatic recrudescences, and two of them terminated fatally. The details of these three cases are as follows:

CASE 1.—J. C., a 13-year-old Italian boy, has been a patient in our clinic for several years and suffered from rheumatic involvement of both mitral and aortic valves. After five weeks of sulfanilamide medication, evidence of congestive failure appeared. The drug was stopped and the boy admitted to the hospital. He died one month after admission in congestive heart failure. Autopsy findings corroborated the clinical diagnosis of chronic rheumatic heart disease with recent reactivation.

CASE 2.—C. C., a 13-year-old Italian girl, had been attending the clinic for several years and presented evidence of mitral insufficiency and stenosis, and of aortic insufficiency. She was given sulfanilamide from September, 1939, through May, 1940, without any untoward effects and showed no evidence of active rheumatic heart disease throughout this period. In November, 1940, she began taking sulfanilamide again and continued doing so until the first week of February, 1941. At this time she complained of headache, chills, and fever. A suspicious petechia was seen on the hard palate. The child was referred to the hospital with the diagnosis of subacute bacterial endocarditis superimposed on rheumatic heart disease. Subsequent laboratory and clinical data confirmed the diagnosis. She died about four months after admission to the ward. No autopsy was obtained.

CASE 3.—The third child (A. F.), a 12-year-old Porto Rican boy, showed evidence of rheumatic activity within one month following cessation of sulfanilamide therapy. He had taken the drug from October, 1940, until the beginning of May, 1941. Three weeks later, the sedimentation rate was found to be elevated and an apical diastolic murmur which had not been heard for more than a year, reappeared.

Among the thirty children in the control group we found three patients with rheumatic reactivation, one of whom died of congestive heart failure.

CASE 1.—S. M., a 14-year-old Italian girl, had mitral insufficiency and stenosis. In August, 1941, she had a sore throat, which was followed shortly by pains in her knees, ankles, and back. These subsided after a few weeks, but recurred in October, 1941. There was, however, no change in either the sedimentation rate or the electrocardiogram.

CASE 2.—K. M., a 14-year-old Porto Rican girl, had rheumatic insufficiency and stenosis. In October, 1940, her sedimentation rate became elevated, and she complained of pains in both knees.

CASE 3.—A. Z., a 13-year-old Italian boy, had mitral insufficiency and stenosis. In February, 1940, evidence of congestive heart failure appeared. This became progressively worse, and he was referred to the hospital. He died March, 1940. No autopsy was obtained.

COMMENT

It is readily seen from the foregoing résumé of our results that we could not demonstrate any prophylactic effect of sulfanilamide in our group. Two deaths and one recrudescence occurred in the treated group; one death and two recrudescences in the control group. Since it is probable that congestive heart failure and subacute bacterial endocarditis in children with rheumatic heart disease indicate the presence of an underlying carditis, our results show therefore that episodes of active rheumatic heart disease occurred with equal frequency in both the treated and control groups.

Nevertheless, we cannot conclude from our study alone, that sulfanilamide has no effect whatever in preventing recurrences of active rheumatic heart disease. Our series is not large enough to be statistically convincing. Furthermore, the general incidence of rheumatic infection during our study period of from September, 1939, through May, 1941, proved to be exceptionally low. It is therefore possible that given a larger number of cases, treated during a time when rheumatic recrudescences were frequent, that the effect of sulfanilamide may have been apparent.

Thomas and her co-workers[3] found that "in no patient under treatment did this serious malady (subacute bacterial endocarditis) develop," and raised the question "of whether sulfanilamide may prevent this complication as well." The course of events in our patient C. C., who developed this complication while regularly taking sulfanilamide, indicates that the use of this drug to prevent subacute bacterial endocarditis leaves much to be desired.

SUMMARY

1. Sulfanilamide in daily doses of 20 grains (1.2 Gm.) was administered to twenty-five ambulatory rheumatic children ranging in age from 6 to 14 years. As controls, we observed thirty children with rheumatic heart disease who were attending the cardiac clinic but who were not given sulfanilamide.

2. Observations were started in September, 1939, and were carried through to May, 1940. They were resumed in September, 1940, and stopped in May, 1941. Two periods each, therefore, of approximately eight months each comprised the time of the study.

3. Minor toxic effects were noted. These consisted of: (1) anorexia, one case. (2) Moderate decrease in hemoglobin content in four cases, accompanied by a drop in red blood count in one of the children. (3) Moderate leucopenia in four patients with concomitant mild granulocytopenia in two of them. The blood counts returned promptly to normal in every instance upon withdrawal of the drug, and in a few cases even while the drug was continued. (4) Mild maculopapular eruption in two patients.

4. Two deaths (one from congestive heart failure, and another from subacute bacterial endocarditis) and one rheumatic recrudescence occurred in the

treated group. One death (from congestive heart failure) and two recrudescences occurred in the control group.

5. In our series of cases sulfanilamide did not appear to be of value in preventing rheumatic recrudescences.

REFERENCES

1. Coburn, A. F., and Moore, L. V.: The Prophylactic Use of Sulfanilamide in Streptococcal Respiratory Infections, With Especial Reference to Rheumatic Fever, J. Clin. Investigation 18: 147, 1939.
2. Thomas, Caroline B., and France, Richard: A Preliminary Report of the Prophylactic Use of Sulfanilamide in Patients Susceptible to Rheumatic Fever, Bull. Johns Hopkins Hosp. 64: 67, 1939.
3. Thomas, Caroline B., France, Richard, and Reichsman, Franjo: The Prophylactic Use of Sulfanilamide in Patients Susceptible to Rheumatic Fever, J. A. M. A. 116: 551, 1941.

CLINICAL CHEMISTRY

THE GLYCOLYTIC ENZYMES OF SYNOVIAL FLUID

ROGER S. HUBBARD, PH.D., AND RICHARD C. PORTER, BUFFALO, N. Y.

IN A previous publication[1] certain actions of glycolytic enzymes in cerebrospinal fluid obtained from patients with epidemic meningitis were discussed. Evidence was presented showing that while the larger part of the reducing sugar, glucose, in such spinal fluid disappears quite rapidly on incubation, the trace of fructose which is present[2] is destroyed very slowly. Other experiments showed that the enzyme which catalyzed the reaction was present in the cellular elements, and indirect evidence was presented which tended to prove that this enzyme was present in the polymorphonuclear leucocytes of the fluid. Further consideration of the question has shown that two objections can be made to these tentative conclusions. The first one is based on the nature of the material studied. The specimens in which the highest concentrations of leucocytes occurred were drawn shortly after acute exacerbations of the infection, and while tests designed to show the presence of meningococci gave negative results, still it is not impossible that organisms were present in small numbers and multiplied upon incubation of the specimens. Since meningococci react more readily with glucose than with fructose or, at least, form acid more readily from the first-named sugar,[4] the results cannot perhaps be attributed with certainty to the polymorphonuclear leucocytes. The second criticism is of an entirely different nature. Willstäter and Rhodewald,[5] in the course of investigations of the mode of formation of lactic acid from sugars, showed that, when buffered with phosphate solutions, glucose and fructose were about equally effective as sources for the formation of glycogen and lactic acid by suspensions of leucocytes.

Since these questions arose in connection with the earlier observations, it seemed desirable to carry out experiments in which (A) there should be available a convenient sterile source of large numbers of human polymorphonuclear leucocytes; (B) the sugars studied should be substances of known purity rather than those occurring in a complex biologic solution; and (C) the cells should be suspended in some biologic medium other than cerebrospinal fluid. In 1928 Cajori and Pemberton[6] described a patient with a sterile inflammatory reaction of the knee joint. On aspiration of this material it was found to be free from organisms but very rich in polymorphonuclear leucocytes. The authors carried out and reported the results of experiments upon the destruction of glucose added to this medium. Recently an opportunity presented itself of studying material from a similar case, and we decided to study the destruction of both glucose and fructose when added to this synovial fluid.

From the University of Buffalo, School of Medicine and the Edawrd J. Meyer Memorial Hospital.

Received for publication, Jan. 19, 1943.

The patient from whom the fluid was obtained was a Negro man 58 years of age. His chief complaint was that a swelling of the right knee was present and that he could move it only with difficulty. The knee was markedly swollen, not tender on palpation, and showed no sign of acute inflammation. The presence of fluid could be demonstrated on physical examination. Apart from this he had no complaints, and there were no other physical or laboratory findings which were abnormal. Serologic tests for gonorrhea and syphilis were negative. Material was repeatedly obtained from the joint space by aspiration. This synovial fluid was rich in protein and contained large amounts of mucin. It was apparently quite rich in fibrin, for in some instances a clot formed before studies could be initiated. Large numbers of leucocytes were present at all times, but the number varied quite markedly in different specimens. In at least one instance more than 1 c.c. of packed cells was obtained by centrifuging 10 c.c. of the fluid. Ninety per cent of the cellular elements were polymorphonuclear cells. The material was repeatedly examined for the presence of mircoorganisms. Tests were made, not only upon each of the six specimens used in the experiments to be discussed below, but also on at least six others. The methods of examination used included direct smears, cultures on many types of media, including those suitable for studying aerobic and anerobic organisms. Methods especially adapated for culturing the gonococcus and the tubercle bacillus were also employed. Specimens were also incubated for periods up to ninety-six hours and the incubated material later examined for organisms by direct smears and inoculation into various types of broth. Specimens of the fluid were also injected into guinea pigs. Positive results were not obtained by any of these methods in any instance. It seems certain that any demonstrable effects of cellular elements which might be found in studying this fluid were due to the leucocytes, for the number of organisms present must have been insignificant. The authors believe that the material was completely sterile.

In carrying out the experimental procedures, six different samples of this synovial fluid were used. The cell contents of these fluids varied markedly, but all were very high. In some studies the cells were first removed by centrifuging, and in others untreated fluid was used. These fluids were divided into portions of approximately 6 c.c. In many instances small amounts, usually 0.6 c.c., of concentrated sterile solutions of the purest glucose or fructose obtainable were added to these 6 c.c. portions and an equal amount of sterile water to an equal volume of the same material. These treated solutions were then mixed as thoroughly as possible to try to prepare equal concentrations of the cellular elements, and divided into three equal parts, each of which contained approximately 2 c.c. One of these was analyzed at once, one was placed in an incubator at 37° C., and the other placed in the icebox. An attempt was made to keep all solutions in each separate one of the six experiments identical, but this was found to be impossible because the leucocytes showed a marked tendency to clump together and because the gelatinous nature of the fluid, due to the presence of mucin, further prevented the preparation of suspensions of cells which were even approximately uniform. Even the three control preparations which made up integral parts of each separate test were not always exact duplicates of each other, although they seemed to show little variation in composition. The incubated and control solutions were precipitated by the zinc sulfate and sodium

hydroxide technique of Somogyi.[7] Reducing compounds were determined by the method of Folin and Wu[8, 9] and modified, when concentrations were low, according to the suggestions of Hubbard and Allison.[10] Fructose analyses were made by the resorcinol technique of Roe.[11] The solutions in which the determinations were finally carried out represented 1 to 10 dilutions of the original material. For the determination of concentration of sugar between 0.1 and 1.0 mg. per 100 c.c. (equivalent to concentrations of from 1 to 10 mg. per 100 c.c. of the original solutions), the method used for the determination of fructose was much more satisfactory than was the one used in determining reducing compounds. In each instance a culture was made of every solution upon which quantitative data were obtained, and all were found to be sterile.

TABLE I

REFERENCE AND EXPERIMENT NUMBER		MATERIAL USED	NOTES	SUGAR ADDED	INCUBATED (HR.)	INITIAL CONTROL (MG. PER 100 C.C.)	ICEBOX CONTROL (MG. PER 100 C.C.)	INCUBATED (MG. PER 100 C.C.)
A	Average 6	As obtained		None	48	67	53	6
	Average 5	As obtained		Glucose	48	162	156	10
	Average 6	As obtained		Fructose	48	81	80	22
B	IV	As obtained		None	24	71	73	7
	IV	As obtained		Glucose	24	175	176	12
	IV	As obtained		Fructose	24	81	87	22
	IV	As obtained		None	48	71*	75*	7*
	IV	As obtained		Glucose	48	155*	173*	12*
	IV	As obtained		Fructose	48	81*	84*	10*
C	VI	As obtained		None	48	51*	47*	20*
	VI	As obtained		Glucose	48	138*	132*	2*
	VI	As obtained		Glucose	48	220	197	116
	VI	As obtained		Fructose	48	80*	79*	56*
	VI	As obtained		Fructose	48	39	39	35
D	IV	As obtained	Incubated for twenty-four hours before experiment	None	24	1	10	12
	IV	As obtained		Glucose	24	105	70	29
	IV	As obtained		Fructose	24	89	87	32
E	I	Centrifuged		None	48	67	65	12
	II	Centrifuged		None	48	81	89	89
	III	Centrifuged		None	48	61	60	60
	IV	Centrifuged		None	48	48	50	46
	V	Centrifuged		None	48	69	64	50
	Average 5	Centrifuged		None	48	65	66	51
	Average 4	Centrifuged		Glucose	48	172	168	176
	Average 5	Centrifuged		Fructose	48	84	84	87
F	IV	Centrifuged	Washed cells added	None	96	45	44	20
	IV	Centrifuged		Glucose	96	146	141	40
	IV	Centrifuged		Fructose	96	73	80	31

The letters are given for reference to the text. Roman numerals show studies upon samples of fluid obtained by each of six separate punctures. Under "glucose" are given figures for total reducing compounds by the Folin and Wu method. Under "fructose" figures determined by the resorcinol reaction.

*Included in the averages listed under A.

The significant results obtained are given in Table I. In section A are given the average values for the studies in which sugars were added to the synovial fluid and the fluids incubated for forty-eight hours. Qualitatively the results of these experiments were all similar, and average values have therefore been presented. Quantitatively the results varied quite markedly. The most extreme variations encountered are given in sections B and C of the table. The average figures show that at least 90 per cent of the sugar originally present and 95 per cent of the glucose added were destroyed, while only between 70 and

75 per cent of the smaller amount of fructose added was lost on incubation. It seems probable that all the actual glucose present was destroyed in these experiments. The difference in the rate of destruction of these two sugars corresponds to the difference observed in the earlier experiments upon spinal fluid but is not as great as that found in those studies. The amount of the added fructose remaining in the solutions in the different experiments varied inversely as the concentrations of the cells present. The total reducing compounds reported as glucose, which were present after incubation, did not appear to be related to the cellular content. We believe that these figures largely represent nonglucose reducing compounds rather than glucose which had not been destroyed and that the irregularities noted are, in part, due to the difficulties inherent in determining such low concentrations of "sugar" and in part due to variations in the amount of some reducing compound extracted from the leucocytes in the different experiments.

Experiment B illustrates the difference in the destruction of these sugars when the solutions were incubated for periods of from twenty-four and forty-eight hours. The specimen studied in this instance was unusually rich in cells. Precautions were taken to try to insure the presence of equal suspensions of cells in all the solutions used, but it is probable that rather marked variations occurred. The added glucose was completely destroyed in twenty-four hours, while significant amounts of fructose, which was present initially in much lower concentration, were still found after forty-eight hours of incubation. The results obtained suggest that fructose was destroyed much more slowly during the second day of incubation than during the first one. This is true whether the figures are expressed in terms of the total amount of sugar or of the per cent of the total sugar which was present at the beginning of each twenty-four-hour period. Whether this apparent decrease in the rate of destruction was brought about by a decreased efficiency of the enzyme system, as some control experiments seemed to indicate, or resulted from a decrease in the amount of substrate, or from both cannot be determined.

Experiment C was designed to test the effect of variation in the amounts of the substrates upon the amounts of the sugars destroyed. In this instance varying amounts of the two sugars were added to a second specimen of fluid, which was much lower in cell content than was that used in Experiment B. In this experiment glucose in a concentration of 100 mg. per 100 c.c., or approximately a total of 2 mg. of sugar, was destroyed by the enzyme in 2 c.c. of fluid in forty-eight hours. Much smaller amounts of added fructose were destroyed. When 1.6 mg. of fructose (2 c.c. of an 80 mg. per 100 c.c. solution) were present, only 0.5 mg. was destroyed; when 0.8 mg. was present, only 0.08 mg. were destroyed. The difference between the reaction of the enzyme to the two sugars was striking, and in this experiment, as well as in the one just discussed (B) the amount of fructose removed appeared to decrease when the amount of sugar was decreased. In this instance also the difference in the rate of destruction was marked, whether it was expressed as amount of sugar or as per cent of the total amount of fructose initially present.

In Experiment D the addition of sugars to a sugar-free synovial fluid was studied. A specimen of fluid very rich in leucocytes (the same material used in experiment B) was incubated for twenty-four hours before the experiment was

begun. This experiment was the second one in which such a procedure was tried. In the earlier attempt all the glycolytic power toward both glucose and fructose disappeared when a speciman of fluid was incubated for one hundred hours. In this instance when sugars were added to the sugar-free fluid, 75 to 85 per cent of the 2 mg. of glucose and approximately 50 per cent of 1.8 mg. of fructose were destroyed in twenty-four hours. This experiment shows clearly that glucose was more rapidly destroyed than fructose when the sugars were added to incubated sugar-free synovial fluid. The destruction of fructose was approximately of the same magnitude as was that observed when fructose was added to the same untreated fluid (see results of Experiment B), but the destruction of glucose was apparently less marked than when the unincubated fluid was used. A number of factors affect the results which make us unwilling to draw definite conclusions from a comparison of these experiments. One of these is the difficulty in obtaining uniform cellular suspension. Another is the important part played in the calculations by the residual reducing compounds present at the time when the final analyses were made. It has already been pointed out that these varied markedly in different experiments, and in the present instance they appear to have been unusually high. Apart from such technical considerations, it is evident that two different effects may have been produced by the preliminary period of incubation. One is a destruction of the glucose originally present in the solution, so that in this instance the added fructose was the only sugar in the incubated material. The other is that the enzymes themselves may have been altered by the treatment, and this change in the enzyme may have had more effect upon the destruction of glucose than of fructose. Attempts were made to study this problem by modifying the conditions in various ways, but it was not found possible to determine definitely the cause of the difference between the results of Experiments B and D.

In Experiment E are given the results obtained by incubating synovial fluid from which cells had been removed by preliminary centrifugalization. In no experiment in which glucose or fructose was added to the cell-free fluid was there a significant change in concentration after incubation, and the average values only, therefore, have been included in Table I. The results of the experiments in which no sugar was added showed variations which were much greater than the probable accuracy of the methods employed, and the results of each have therefore been included. It is evident that not only were the average changes produced by incubation of the cell-free fluid smaller than those produced when cells were present, but also that in separate experiments the differences were marked. This seems to prove that the glycolytic enzymes affecting both glucose and fructose were present in the polymorphonuclear cells. However, two of a total of fourteen specimens (two of five specimens to which sugars were not added) showed a significant destruction of reducing compounds after forty-eight hours of incubation, and in one of these two instances, Experiment I. this destruction was marked. It seems most probable to us that in these two specimens enzymes had been extracted from the leucocytes, but the action of some other source of the glycolytic activity cannot be excluded. It should be noted that in the studies of Cajori and Pemberton[6] glycolytic activity of cell-free synovial fluid was demonstrated and that this activity was much less marked than was that of the uncentrifuged material.

In Experiment F are given results obtained when cells were removed from a speciman of fluid, washed in normal salt solution, and resuspended in the supernatant fluid. This experiment is interesting for a number of reasons. First, on the readdition of cells to a cell-free synovial fluid which possessed no glycolytic activity (as a control, see Experiment E IV, Table I), a distinctly demonstrable glycolytic activity was produced. Second, the destruction of glucose was more marked than was the destruction of fructose. Third, the process of centrifuging, washing and resuspending the cells had greatly reduced their enzymatic power, for the destruction of glucose and fructose was markedly less than the destruction in uncentrifuged material (see experiment C IV as control, Table I) although precautions were taken to have the concentration of the resuspended cells greater than that found in the original solution. In a second similar experiment carried out for forty-eight instead of ninety-six hours, no destruction of fructose and only a relatively slight destruction of glucose were observed.

Besides the experiments described above, cells from four of the six specimens, including those used in Experiment IV, were washed, resuspended in 0.85 per cent salt solution, and incubated for periods varying from forty-eight to ninety-six hours. No satisfactory evidence of a destruction of glucose or fructose added to such solutions was obtained in any of the experiments. This was probably due, in part, to the lessening of the enzymatic activity brought about by washing the cells illustrated by the experiments discussed in the preceding paragraph and in part by the unsuitability of the medium in which the cells were suspended. The suspensions to which no sugars were added did, however, give some fairly interesting results. When unincubated, sugar-free cellular suspensions were studied, no positive reducing or resorcinol reaction was obtained. After incubation the average "glucose" content of the four preparations was 12 mg. per 100 c.c., and the "fructose" concentration was 1.3 mg. per 100 c.c. The values in the specimens incubated ninety-six hours were higher than those studied after forty-eight hours. We believe that these apparent changes in the "sugar" content, which were almost certainly not due to glucose and fructose, were produced by disintegration of the cells. The same conclusion probably applies, we believe, to the small amounts of reducing compounds found in some of the experiments described in the earlier part of this paper.

It seemed possible that evidence might be obtained showing that glucose was changed into fructose or that fructose was converted into glucose; in other words, that a reaction simulating the Lobry de Bruyn effect[12] could be demonstrated during glycolysis by polymorphonuclear leucocytes. All specimens, both those containing glucose and fructose, as well as all control solutions, were therefore analyzed by both the reducing technique of Folin and Wu and the resorcinol technique of Roe. No evidence of the formation of one sugar from another was obtained. All variations of this type which were observed were of a magnitude which could be properly explained by the gradual increase in the control values found when suspensions of washed cells were incubated. Of course, the formation of small amounts of glucose from fructose under conditions which would lead almost immediately to their degradation would not be detected in such experiments.

CONCLUSIONS

When glucose or fructose was added to sterile synovial fluid which was rich in polymorphonuclear leucocytes, the sugars were destroyed. The rate of the destruction of glucose was at least twice as great as was that of fructose. The enzymes were present almost wholly in the cells, although they could occasionally be demonstrated in the cell-free fluid. The results confirm qualitatively those previously obtained upon cerebrospinal fluid.

The authors wish to express their gratitude to Mrs. Elfriedt Fendt Sicari for her technical assistance in the bacteriologic phases of this study.

REFERENCES

1. Hubbard, R. S., Russell, N. G., Jr., and Russell, N. M.: Sugars and Glycolytic Enzymes of Spinal Fluid in Epidemic Cerebrospinal Meningitis, J. Clin. Investigation 15: 519, 1936.
2. Hubbard, R. S., and Garbutt, H. R.: Resorcinol (Fructose) Reaction in Cerebrospinal Fluid, Proc. Soc. Exper. Biol. & Med. 32: 986, 1925.
3. Hubbard, R. S., and Russell, N. M.: The Fructose Content of Spinal Fluid, J. Biol. Chem. 119: 647, 1937.
4. Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P.: Bergey's Manual of Determinative Bacteriology, ed. 5, Baltimore, William & Wilkins Co., p. 280.
5. Willstäter, and Rhodewald, M.: Ueber Aufbau und Abbau des Glykogens durch Leukocyten, Hoppe-Seyler's Ztschr. f. physiol. Chem. 247: 115, 1937.
6. Cajori, F. A., and Pemberton, R.: The Chemical Composition of Synovial Fluid in Cases of Joint Effusion, J. Biol. Chem. 76: 471, 1928.
7. Somogyi, M.: A Method for the Preparation of Blood Filtrates for the Determination of Sugar, J. Biol. Chem. 86: 655, 1930.
8. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. 38: 81, 1919.
9. Folin, O., and Wu, H.: A System of Blood Analysis. Supplement I. A Simplified and Improved Method for the Determination of Sugar, J. Biol. Chem. 41: 367, 1920.
10. Hubbard, R. S., and Allison, C. B.: Residual Reduction in Blood Filtrates After Treatment With the Colon Bacillus, Proc. Soc. Exper. Biol. & Med. 25: 408, 1928.
11. Roe, J. H.: A Colorimetric Method for the Determination of Fructose in Blood and Urine, J. Biol. Chem. 107: 15, 1934.
12. Armstrong, E. F., and Armstrong, K. F.: The Carbohydrates, ed. 5, New York, 1934, Longmans, Green & Co., p. 47.

STUDIES OF PANCREATIC FUNCTION*

II. The Effect of Injury to the Pancreas or the Liver Upon the Amylase and Lipidase Content of the Blood

Joseph H. Roe, Ph.D., and Norman P. Goldstein, M.A.
Washington, D. C.

QUESTIONS concerning the nature and origin of the lipidolytic enzymes of the blood are problems of major interest in physiology and clinical laboratory science. The earliest work of this kind was that of Hewlett[1] in 1904, who reported that after pancreatic injury there was in the urine an increase in the content of an enzyme which splits ethyl butyrate, or an "esterase"; also in one case there was observed in the urine an enzyme which splits the fats of olive oil, or a "lipase." Later, von Hess[2] (1912), also Hiruma[3] (1923), using

*From the Department of Biochemistry, School of Medicine, The George Washington University.
Received for publication, May 14, 1943.

ethyl butyrate as a substrate, obtained evidence indicating that there is an increase in the esterase content of blood after pancreatic duct ligation. In 1932 Cherry and Crandall[4] reported that after ligation of the pancreatic ducts in dogs, they could demonstrate in blood the presence of an olive oil-splitting enzyme (lipase), which normally did not appear in the blood stream. They also found that the activity of an enzyme normally present in blood, as measured by the hydrolysis of ethyl butyrate or tributyrin (esterase), did not show uniform increases after pancreatic duct ligation. They, therefore, concluded that the appearance in the blood of an enzyme which splits olive oil (lipase) is a specific indication of pancreatic injury.

Cherry and Crandall suggested that the term "lipase" be used to designate an enzyme which splits triglycerides of long chain fatty acids, as are in olive oil, and that the term "esterase" be used for an enzyme which splits simple esters, such as ethyl butyrate. They found, however, that tributyrin is apparently split by the same enzyme that hydrolyzes ethyl butyrate.

In 1934 Comfort and Osterberg,[5] using the Cherry and Crandall method for estimating serum lipase, reported elevated values for serum lipase in 95 per cent of cases of active pancreatitis, in 36.5 per cent of cases of carcinoma of the pancreas, and in 60 per cent of cases of carcinoma of the ampulla of Vater. In a later paper[6] these authors reported essentially the same results with this test, but in addition they found an increased serum lipase content in 97 per cent of cases of disease of the biliary tract in which there was an associated pancreatitis. These authors stress the requirement that the test must be made in less than ten days after the onset of pain. They conclude that the serum lipase determination is an efficient test for active pancreatitis, but less so for malignant disease of the pancreas.

In another paper[7] we have reported a procedure which includes greatly improved conditions for the determination of the lipidolytic enzymes of blood serum. With this method we have carried out an investigation in which conditions were set up to study the origin of these enzymes and their possible relation to pancreatic disease. Variations in the lipidolytic enzymes of the blood were followed with our method using five different substrates. Since Elman,[8] and others, have found the blood amylase a useful determination in acute pancreatic disease, it was decided to make parallel determinations of the serum amylase along with the lipidase estimations. For the amylase determination we used the method of Somogyi.[9] The cat was used as the experimental animal.

Four general types of experiments were set up. These were a study of the effect of the following upon the content of amylase and lipidolytic enzymes in the blood: (1) eserine and mecholyl stimulation; (2) ligation of the pancreatic ducts plus eserine and mecholyl stimulation; (3) pancreatectomy; (4) liver poisoning by chloroform. We were particularly interested in finding what conditions will elevate the blood concentration of these enzymes; whether, or not, these enzymes have an origin other than in the pancreas. Also, by means of the five substrates used, we hoped to obtain data showing which substrate could be considered the most reliable for studying pancreatic disease. A discussion of the experimental techniques and results is given.

ESERINE AND MECHOLYL STIMULATION

Experiments with eserine and mecholyl were carried out to standardize a method for stimulating pancreatic secretion which could be used for further experimentation. The procedure adopted was as follows: Cats were anesthetized by injecting intraperitoneally 30 mg. of nembutal per kilo of body weight. After anesthesia had developed, a control sample of about 18 c.c. of blood was collected by heart puncture. Eserine sulfate and mecholyl were then injected subcutaneously. A single dose of eserine sulfate, ranging from 0.125 to 0.50 mg., was administered, and the dosage of mecholyl was 0.05 mg. per kilo of body weight injected every half hour. After three to four hours of mecholyl administration a second sample of 18 c.c. of blood was collected from the heart.

Large samples of blood were collected to be able to carry out tests on five substrates for the lipidolytic enzymes, and also to carry out an amylase test. Since large blood samples had to be taken, it was obvious that only two samples of 18 c.c. each should be studied with an animal the size of the cat to obtain valid data; otherwise the blood analyses might reflect the result of hemorrhage. Therefore, in most of the data of this report only two blood samples were analyzed, the control and the experimental collected at the end of the experiment.

In Table I are the results of mecholyl stimulation in the presence of inhibition of acetyl choline esterase action by eserine. In practically every group of comparative analyses there is an increase in the amount of amylase and lipidolytic enzymes in the blood serum. A control on this experiment would be useful as some blood volume changes might take place as a result of the initial bleeding, the salivation, or other losses of fluids. The nearest to a control that could be obtained is the experiment with Cat 21, a depancreatized animal that had been healed with insulin. The increases observed in this cat are 12 per cent with tributyrin as substrate, 10 per cent with triolein, and 14 per cent with olive oil, values which are within the limits of error of this method. In view of these results with a depancreatized animal, it appears that the increases in blood enzymes observed after eserine and mecholyl stimulation of the intact animals are significant and that they reflect principally a pancreatic response. The increases in the lipidolytic enzymes noted are essentially parallel with the different substrates; hence no differentiation in these enzymes could be made. It is also of interest that the increases in amylase and lipidase are of the same magnitude.

PANCREATIC DUCT LIGATION WITH MECHOLYL STIMULATION

The results of experiments with pancreatic duct ligation are shown in Table II. In these experiments cats were anesthetized with nembutal and a control sample of blood was taken from the heart. A laparotomy was performed, and the pancreatic ducts were doubly ligated and cut between the ligations. Care was taken to ligate all ducts from the pancreas and not to obstruct the bile ducts. The abdomen was closed with sutures and skin clamps. Eserine and mecholyl were administered as described above. After two to five hours of mecholyl administration samples of blood were collected. All bloods were analyzed for amylase and for lipidolytic enzymes with five substrates.

TABLE I

THE EFFECT OF ESERINE AND MECHOLYL STIMULATION ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD OF THE CAT

CAT NO.	EXPERIMENT SUBCUTANEOUS INJECTION OF	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
9	Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 15 min.			140	180	76	93					39	66
	Same, repeated one day later			120	149	69	84	68	95			32	46
16*	Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 30 min.	912	1180	76	92	32	43	35	54			8	27
20	Eserine sulfate, 0.25 mg.; mecholyl, 0.05 mg. per kg. per 30 min.	1472	1938	93	112			51	88	35	47	27	56
22	Eserine sulfate, 0.25 mg.; mecholyl, 0.05 mg. per kg. per 30 min.	676	1336	77	100	43	56	51	49	12	27	9	20
21	(Depancreatized cat.) Eserine sulfate, 0.125 mg.; mecholyl, 0.05 mg. per kg. per 30 min.			123	141					45	50	42	49

*In experiment with Cat 16 experimental blood sample was removed two hours after start of injections.

For amylase values the Somogyi unit was used. The lipidolytic enzyme unit used here has been defined in another paper.[7] Values are expressed in units per 100 c.c. of serum. The experimental blood sample was collected approximately 3.5 hours after starting the injections.

TABLE II

THE EFFECT OF PANCREATIC DUCT LIGATION AND MECHOLYL STIMULATION ON THE CONTENT OF AMYLASE AND LIPOLYTIC ENZYMES OF THE BLOOD OF THE CAT

CAT NO.	EXPERIMENT	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
10	Pancreatic ducts ligated. Blood sample 24 hr. postoperative	420	1056	143	176	93	99	110	139			32	65
15	Pancreatic ducts ligated. Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 30 min. Blood sample 2 hr. postoperative	1057	1950	121	147	64	92	92	74			34	82
	Blood sample 5 hr. postoperative	1057	2050	121	443	64	111	92	374			34	109
19	Pancreatic ducts ligated. Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 30 min. Blood sample 3.5 hr. postoperative			111	251					30	177		
23	Pancreatic ducts ligated. Eserine sulfate, 0.125 mg.; mecholyl, 0.05 mg. per kg. per 30 min. Blood sample 3.5 hr. postoperative	1195	1445	109	200	44	86	70	121	34	79	28	69

Values are expressed in units per 100 c.c. of serum. For amylase the Somogyi unit was used. The lipidolytic unit used has been defined in another paper.[7]

TABLE III

THE EFFECT OF PANCREATECTOMY ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD

CAT NO.	TIME AFTER PANCREATECTOMY BLOOD SAMPLE WAS COLLECTED	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
27	24 hours postoperative	976	911	108	102	57	44	73	67			25	31
29	4 days postop.	1726	644	99	384	53	96	76	280	31	101	26	106
	9 days postop.	1726	835	99	86	53	42	76	77	31	16	26	12
12	12 days postop.		1113		61		56		34				168
	19 days postop.		872		148		44		73				65
13	14 days postop.		180		59		40		47				30
	28 days postop.		1184		115		50		55				24
	42 days postop.		1075		135		59		91				35
	56 days postop.		1113		116		38		70				30
	70 days postop.		1035		141		81		97				59

Values are expressed in units per 100 c.c. of Serum. For amylase the Somogyi unit was used. The lipidolytic unit has been defined in another paper.[7]

This experiment was done to simulate conditions that probably exist in an active pancreatitis where obstruction occurs. A marked increase in amylase and lipidase was observed. The increases in amylase and lipidase are of essentially the same magnitude. These experiments show that pancreatic enzymes readily pass into the blood stream when the flow of external secretion of the pancrease into the duodenum is blocked. They do not indicate any difference in sensitivity of response of amylase as compared with lipidase. With the five substrates for lipidolytic enzymes, the order of sensitivity of response was the same as in normal cat serum. Tributyrin was split most rapidly; benzyl butyrate was next; and ethyl butyrate, olive oil, and triolein were the least sensitive to the splitting action of the enzymes present. Thus, a differentiation in the lipidolytic enzymes that appear in the blood serum after pancreatic duct ligation is not shown by these experiments.

THE EFFECT OF PANCREATECTOMY

The origin of the amylase and lipidase of the blood is a question of considerable importance. Somogyi[9] has presented convincing evidence that amylase is produced by the liver and it is well known that amylase is secreted by the salivary glands. The effect of pancreatectomy on the serum amylolytic potency has been investigated by a number of laboratories.[10-13] Reports upon this work are in good agreement in regard to a sharp postoperative drop in serum amylase concentration, but there are conflicting results as to whether or not the serum amylase value returns to normal with control of the diabetes by insulin. In as far as we know, the effect of pancreatectomy upon the concentration of lipidolytic enzymes in the blood has not been reported. We, therefore, decided that a study of the concentration of serum lipidase and amylase in cats following pancreatectomy would be of interest.

Pancreatectomy was performed under nembutal anesthesia, and the diabetes was controlled by insulin administration. The data obtained in these experiments are shown in Table III. The results in the experiment with Cat 27, in which blood was collected twenty-four hours after pancreatectomy, do not show variations that could be considered outside the limits of experimental error. Considering the other experiments as a group, the data show a decrease in the values for amylase and lipidolytic enzymes, following pancreatectomy, with a mixed return trend toward preoperative levels. No conclusion seems warranted from these results other than the very definite observation that amylase and lipidolytic enzymes do not disappear from the blood serum after pancreatectomy. This observation is in agreement with other work regarding the effect of pancreatectomy on the amylase content of serum and it appears to be a new finding concerning the lipidolytic enzymes of the blood.

The results of these studies, together with those of Table II, lead to the conclusion that the lipidolytic enzymes of the blood have an extrapancreatic as well as a pancreatic origin.

CHLOROFORM POISONING

It has been reported by several laboratories[4, 14, 15] that the liver does not produce an enzyme which splits the lipids of olive oil. We have obtained in-

TABLE IV

THE EFFECT OF CHLOROFORM POISONING ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD OF THE CAT

CAT NO.	HOURS AFTER INJECTION OF CHLOROFORM BLOOD SAMPLE WAS COLLECTED	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
17	18	1179	533	93	153	54	102	79	144			31	29
21	20	710	428	150	243	108	257	101	217	50	38	45	31
18	42	755	527	99	126	41	55	64	81			23	17

Dosage: 1 c.c. U.S.P. Chloroform per kg. of body weight. Values are expressed in units per 100 c.c. of serum. For amylase the Somogyi unit was used. The lipidolytic unit used has been defined in another paper.[7]

formation in agreement with this finding: a glycerol extract of liver was found to split ethyl butyrate, benzyl butyrate, and tributyrin, but not olive oil. In view of these findings, it was believed desirable to carry out liver poisoning experiments which might offer suggestions upon the influence of the liver, when diseased, upon the concentration of lipidolytic enzymes in the blood. The use of chloroform seemed indicated, since this compound has a primary toxic effect upon the liver.

In these experiments cats were anesthetized with nembutal, and control samples of blood were collected from the heart. Chloroform dissolved in olive oil was injected subcutaneously, the dosage being 1 c.c. of U.S.P. reagent per kilo of body weight. Samples of blood were collected at times varying from eighteen to forty-two hours after injection of chloroform. All bloods were analyzed for amylase and for lipidolytic enzymes by five substrates.

The results are shown in Table IV. The serum amylase decreased in all cases. The triolein and olive oil-splitting enzymes showed a very slight decrease, the significance of which is uncertain. A marked increase in the enzyme, or enzymes, of serum which split tributyrin, ethyl butyrate, and benzyl butyrate occurred. These results are of considerable interest because they show a differentiation in the lipidolytic enzymes of the blood; in an experimental procedure upon the intact animal, the enzyme, or enzymes, which split esters and tributyrin (esterase), were found to be increased in the blood serum, while the enzyme, or enzymes, which split triolein and the lipids of olive oil (lipase), remained at approximately the same or a slightly lower level. This is good evidence in favor of the assumption that there are in the blood two lipidolytic enzymes, or groups of enzymes: namely, esterases, which split simple esters and short chain fatty acid triglycerides, and lipases which hydrolyze triglycerides.

The most obvious conclusion concerning the increase in the esterase activity of the serum in chloroform poisoning is that it is a response to liver damage, although chloroform may have a toxic effect upon other organs or tissues which would be reflected by changes in the enzyme concentration of the blood. More work upon this phase of the problem should lead to interesting results.

DISCUSSION

The experiments described above were designed to get information upon the origin of the amylolytic and lipidolytic enzymes of the blood, and to study the relation of the pancreas and the liver to the circulating level of these enzymes in the blood. It was also thought that the use of five substrates for the estimation of the lipidolytic enzymes might yield evidence concerning the identity of these enzymes.

Stimulation with mecholyl in the presence of eserine, with and without pancreatic duct ligation, caused marked increases in the amylolytic and lipidolytic enzymes of the blood of cats. The increases in amylase and in lipidase were of the same magnitude, and there was no essential difference in the responses measured by the five different substrates for the lipidolytic enzymes. These results demonstrated that there is an increase in enzymes of pancreatic origin in the blood when a strong vagal stimulus is applied and when pancreatic duct occlusion occurs.

Pancreatectomy experiments resulted in some decrease in amylase and lipidolytic enzymes in the serum with irregular return trends toward normal levels. These experiments showed that both the "esterase" and "lipase" types of enzymes in the blood of the cat have an extrapancreatic source in addition to their pancreatic origin.

Experiments in which cats were poisoned with chloroform showed a decrease in serum amylase and a very slight decrease in the enzyme, or enzymes, of the serum which split triolein and the lipids of olive oil. Sera from the same animals, however, showed an increase in the enzymes which split tributyrin, ethyl butyrate, and benzyl butyrate. These experiments gave data which indicate a clear differentiation between the so-called "esterase" and "lipase" of the blood serum. Since chloroform has a primary toxic effect upon the liver, these results raise the question of whether or not the "lipase" determination, published by ourselves and others, might reflect the presence of liver disease as well as pancreatic injury. This question may be answered only by clinical studies.

SUMMARY

1. Experiments upon cats have been carried out which were designed to obtain information upon the origin and identity of the lipidolytic enzymes of blood serum.

2. Stimulation with mecholyl in the presence of eserine, with and without pancreatic duct ligation, caused marked increases in the concentrations of amylolytic and lipidolytic enzymes in the blood.

3. Pancreatectomy resulted in postoperative decreases in serum amylase and lipidase, with mixed return trends toward normal levels. These experiments demonstrated that the amylolytic and lipidolytic enzymes of the blood have an extrapancreatic source in addition to their pancreatic origin.

4. Chloroform poisoning caused a decrease in serum amylase. With respect to the lipidolytic enzymes of the serum, a dissociation of effects was observed: The triolein and olive oil-splitting enzymes showed a very slight decrease; and the enzyme, or enzymes, which split ethyl butyrate, benzyl butyrate, and tributyrin, were definitely increased. The latter behavior is evidence in favor of the existence of both "esterase" and "lipase" in blood serum.

REFERENCES

1. Hewlett, A. W.: On the Occurrence of Lipase in the Urine as a Result of Experimental Pancreatic Disease, J. Med. Research 1: 377, 1904.
2. Von Hess, C. L.: Contributions to the Physiology of Lymph. XVIII. The Relation of the Pancreas to the Lipase of the Blood and the Lymph, J. Biol. Chem. 10: 381, 1911-12.
3. Hiruma, K.: Upon the Question of the Origin of Lipase in Blood, Biochem. Ztschr. 139: 336, 1923.
4. Cherry, I. S., and Crandall, L. A., Jr.: The Specificity of Pancreatic Lipase: Its Appearance in the Blood After Pancreatic Injury, Am. J. Physiol. 100: 266, 1932.
5. Comfort, M. W., and Osterberg, A. E.: Lipase and Esterase in the Blood Serum: Their Diagnostic Value in Pancreatic Disease, J. Lab. & Clin. Med. 20: 271, 1934.
6. Comfort, M. W., and Osterberg, A. E.: The Value of Determination of the Concentration of Serum Lipase and Serum Amylase in the Diagnosis of Disease of the Pancreas, Proc. Staff Meet., Mayo Clinic 15: 427, 1940.

7. Roe, J. H., and Goldstein, N. P.: Studies of Pancreatic Function. I. The Determination of the Lipidolytic Enzymes of Blood Serum, J. Lab. & Clin. Med. 28: 1368, 1943.
8. Elman, R.: Surgical Aspects of Acute Pancreatitis, J. A. M. A. 118: 1265, 1942.
9. Somogyi, M.: Blood Diastase as an Indicator of Liver Function, Proc. Soc. Exp. Biol. Med. 32: 538, 1934.
10. Schlesinger, W.: Upon the Origin of Diastatic Ferments in the Blood and Their Indication in Diabetes Mellitus, Deutsche. med. Wchnschr. 34: 593, 1908.
11. Markowitz, J., and Hough, H. B.: A Study of Blood Diastases in Depancreatized Dogs, Am. J. Physiol. 75: 571, 1925-26.
12. Reid, E., Quigley, J. P., and Myers, V. C.: Studies on Animal Diastases. V. Blood and Tissue Diastases, With Special Reference to the Depancreatized Dog, J. Biol. Chem. 99: 615, 1932-33.
13. Friedman, I., and Thompson, W. R.: Induced and Spontaneous Changes in Blood Amylase Particularly in Relationship to the Pancreas, Ann. Surg. 104: 388, 1936.
14. Sobotka, H., and Glick, D.: Lipolytic Enzymes. I. Studies on the Mechanism of Lipolytic Enzyme Action, J. Biol. Chem. 105: 199, 1934.
15. Balls, A. K., and Matlack, M. B.: The Enzymic Hydrolysis of Benzyl Stearate and Benzyl Butyrate, J. Biol. Chem. 125: 539, 1938.

INTRAVENOUS INJECTIONS OF SOLUBLE TIN COMPOUNDS*

Joseph Seifter, M.D., and Edward S. Rambousek, M.D.†
Cleveland, Ohio

THERE are no complete studies of the distribution of tin in the organs following intravenous injection of tin compounds. Accordingly, we adapted for toxicologic study the toluene 3,4-dithiol method developed by Clark[1] and extended to canned foods by DeGiacomi.[2] We also found it necessary to prepare tin compounds suitable for intravenous injection. The results of these studies are reported in this paper.

METHOD OF DETERMINING TIN

Preparation and Storage of Toluene-3-4-dithiol.—We obtained the compound, which is not on the market, by first preparing sizable batches of sodium toluene-3,4-disulfonate (3) and, then as the occasion arose, by converting small amounts of this to the dithiol (3). The distilled product was stored in a dark refrigerator.

Dithiol Solution.—A 0.2 per cent solution of dithiol in 1 per cent aqueous NaOH containing 0.5 per cent thioglycollic acid remains clear for several days when kept in the refrigerator. The solution should be discarded when turbidity appears.

Standard Sn Solutions.—Reagent grade tin metal is dissolved in concentrated HCl containing 0.5 per cent thioglycollic acid so that 1 c.c. represents 1 mg. Sn. This stock solution keeps indefinitely. For use in the analytic procedure, the stock solution is diluted with 0.5 per cent thioglycollic acid in dis-

*From the Department of Pharmacology of the School of Medicine, Western Reserve University.

†Crile Scholar during the summers of 1940 and 1941.

Aided by a grant from the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the American Medical Association. A preliminary communication was presented to the Society for Experimental Biology and Medicine, Cleveland, Ohio, November 12, 1941.

Received for publication, May 4, 1943.

tilled water so that 1 c.c. represents 0.01 mg. Sn. This solution should be prepared fresh each day.

Range of Color.—In the range of 2 gamma to 20 gamma Sn there is a progressive increase in color, and the unaided eye readily detects differences of 3 gamma. In our experiments this sensitivity is 5 times greater than with the molybdenum-blue method.[4]

Procedure.—Fresh tissue (5 to 100 Gm.) is dissolved in a minimum of concentrated HNO_3, evaporated to dryness, and placed in a muffle oven at 500 degrees Centigrade until the charred organic matter is removed (usually twelve to sixteen hours). The ash is brought to fuming with 5 c.c. concentrated H_2SO_4, and heating is continued until solution takes place. After cooling, the mixture is neutralized with NH_4OH, acidified with HCl, diluted to 100 c.c. with distilled water, and finally brought to proper acidity by adding 2 c.c. of concentrated HCl. Hydrogen sulfide is passed through the mixture for 1½ hours, and the precipitate is collected on a Gooch crucible. The precipitating flask is rinsed with 30 c.c. of 10 per cent aqueous NaOH followed by distilled water. Each rinse is passed through the crucible. HCl is permitted to seep through until the filtrate is acid to litmus; then distilled water is passed through. The final volume is adjusted by evaporation or dilution to contain not less than 0.5 gamma nor more than 100 gamma Sn per c.c. To an aliquot of 5 to 10 c.c. in a tube there are added 1 drop of thioglycollic acid, 0.5 c.c. concentrated HCl, and 0.5 c.c. of dithiol reagent. A like amount of tin standard solution is treated in the same fashion. Both tubes are immersed in boiling water for ten seconds and the colors are compared directly by light reflected from a white background. The red color is discharged by various organic solvents (i-amyl acetate, i-amyl alcohol, n-butyl alcohol, di-n-butyl ether, carbon disulfide).

Factors Influencing Amount of Sn Recovered.—1. *Muffle Heating* for twelve to sixteen hours at 500 degrees Centigrade in the presence of H_2SO_4 or NaOH did not result in loss of Sn. The presence of HNO_3 resulted in lower values which were restored to practically complete recovery by heating the ash with concentrated H_2SO_4 to fuming, then neutralizing with alkali, and finally acidifying with HCl. The recoveries were made on duplicate samples in the range of 5 gamma to 100 gamma Sn.

2. *Sulfide Precipitation* resulted in a loss of Sn. With 50 gamma to 1 mg. the recovery was 96 to 100 per cent; at 10 gamma the recovery was 80 to 90 per cent; at 5 gamma it was 60 to 70 per cent.

3. *Presence of Animal Tissue* accounted for still greater loss of Sn in the lower ranges. With 100 gamma to 2 mg. the recovery was practically complete; with 50 gamma it was 90 to 94 per cent; with 5 to 30 gamma it was 60 to 80 per cent. Consequently the method has only qualitative significance if the sample contains 50 gamma or less and is doubtful at 5 gamma.

PREPARATION OF TIN COMPOUNDS

Stannic Citric Acid was prepared by adding 5 c.c. stannic chloride (anhydrous) to 30 Gm. of sodium citrate .11 H_2O dissolved in 40 c.c. of distilled water. The reaction product was precipitated by the addition of 10 volumes of 95 per cent ethyl alcohol. In one hour this was filtered and the white precipitate repeatedly washed with 95 per cent alcohol. Analysis by the dithiol method gave 20 per cent Sn. The product formed aqueous solutions readily

Table I

Distribution of Tin in Organs of Rabbits

NO.	MG. SN INJECTED		DEATH	KIDNEY				LIVER					LUNG					SPLEEN				
	TOTAL	PER KG.		MG. SN	% OF INJ. SN	CONC.	RQ	MG. SN	% OF INJ. SN	CONC.	RQ	% OF KID.	MG. SN	% OF INJ. SN	CONC.	RQ	% OF KID.	MG. SN	% OF INJ. SN	CONC.	RQ	% OF KID.
1	260	106	*	6.00	2.3	38.20	.36	5.11	2.0	4.30	.04	11.2	30.0	11.5	98.7	.93	258	.13	0.05	14.44	.136	37.8
2	250	104	*	8.33	3.3	45.51	.44	4.67	1.6	3.68	.03	8.1	4.1	1.6	26.4	.25	58	.20	0.68	15.38	.147	33.7
3	350	175	2d	83.3	23.0	474.0	2.7	4.41	1.2	4.91	.03	1.0	-	-	-	-	-	.26	0.07	16.25	.092	3.4
4	175	50	4d	7.5	4.2	37.50	.75	13.0	7.3	8.12	.17	21.6	0.80	0.4	2.66	.05	7	1.5	0.85	75.00	1.50	200.0
5	150	50	4d	7.5	5.0	25.00	.50	11.7	7.8	9.00	.18	36.0	1.50	1.0	3.95	.08	15.8	2.0	1.33	80.00	1.60	320.0
6	45.5	25	K 3W	0.43	0.9	3.43	.14	0.53	1.1	1.78	.07	51.8	0	0	0	0	0	.29	0.60	16.11	0.64	468.0
7	13.8	5	K 4W	0.05	.36	0.38	.08	0.31	2.2	0.26	.05	68.4	0	0	0	0	0	.003	0.02	0.31	0.06	81.0
8	15	5	K 4W	0.12	.80	0.81	.16	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0

1	Bladder urine 10.2;	Bile 5.4;	Small Intestine 7.59;	Colon 5.47	Mg. Sn per 100 Gm. fresh organ
2	-	9.50	-	7.38	Mg. Sn per 100 Gm. fresh organ
3	-	0	1.40	1.29	Mg. Sn per 100 Gm. fresh organ
7	-	0	-	-	Mg. Sn per 100 Gm. fresh organ
8	-	0	-	-	Mg. Sn per 100 Gm. fresh organ

Abbreviations: Conc. = Mg. Sn per 100 Gm. of fresh organ.
RQ = Conc/Mg. Sn per Kg. of animal.
Kid. = Kidney.
* = End of injection.
d = days.
K = Sacrificed.
W = Weeks.

from which the sulfide was precipitated very slowly with H_2S. The aqueous solution (12.5 per cent) was acidic and 1 c.c. required 5.72 c.c. of N/10 NaOH for neutralization. For injection only neutralized solutions were used.

Stannous Citric Acid (20 per cent Sn), prepared with stannous chloride and precipitated as just described, flocculated when neutralized. Consequently the acid form was injected.

INTRAVENOUS INJECTION IN RABBITS

Stannic and stannous citric acids were injected instead of tin salts since the latter are liable to produce colloidoclastic shock. Neither compound in solution flocculated with rabbit serum, 10 per cent egg white solution, nor with Ringer's solution at pH 6.0, 7.0, or 8.5. Neither did they hemolyze mammalian erythrocytes.

Death usually occurred during intravenous injection of solutions of either compound in about twenty minutes; when approximately 500 mg. (100 mg. Sn) per kg. had been introduced at the rate of 0.5 c.c. per minute. Tremors appeared when 25 per cent of the fatal dose had been administered; flaccid paralysis when 50 to 60 per cent had been administered; and terminal tonic and clonic convulsions toward the end of the injection. The viscera, especially the lungs, were severely congested. The acutely fatal dose is practically the same as for sodium citrate. Animals which received only 50 per cent of the acutely fatal dose responded with a brief period of excitement followed by lethargy, from which they recovered and showed no further symptoms until death four days later. At autopsy the kidneys were pale and granular. No edema was seen. Animals which received 25 per cent or less of the acutely fatal dose survived until sacrificed about one month later.

ORGAN CONCENTRATION AND FATE OF THE INJECTED TIN

Our data in Table I are too few to warrant detailed deductions but point to the similarity of tin to heavy metals,[4, 5] especially bismuth. In general the amounts of Sn in the kidneys, and lungs, the intestines, and the bile diminished progressively with the time after injection; but those in the livers, and the spleens increased before they decreased. Animals that died during injection, or shortly after, had Sn in all organs and fluids that were examined; but after three weeks only spleen, liver, and kidney had amounts sufficient for analysis. The kidney probably loses the metal last. The kidneys, except in Rabbit 1, had a considerably greater concentration of metal than did other organs. The concentrations in the intestines and the bile did not exceed those of the urine. The spiral valves of the intestines were not involved in detoxification since they did not have high tin concentrations and had no deposit of tin sulfide.

CONCLUSIONS

1. Dithiol is applicable for quantitative analysis of tin in tissues.

2. The method is quantitative for samples containing at least 50 gamma of tin.

3. The sojourn and concentration of tin resemble heavy metals, especially bismuth.

4. The organs tolerate relatively high concentrations of tin.

5. Tin is excreted rapidly following intravenous injection.
6. Stannic citric acid and stannous citric acid possess low toxicity.

REFERENCES

1. Clark, R. E.: The Detection and Colorimetric Determination of Tin by Means of Substituted 1:2 Dimercaptobenzenes. A Specific Reagent for Tin, Analyst 61: 242, 1936.
2. DeGiacomi, R.: The Determination of Tin in Foodstuffs by Means of Dithiol, Analyst 65: 216, 1940.
3. Mills, W. H., and Clark, R. E.: Stereochemistry of Some New Complex Thio Salts of Mercury, Cadmium and Zinc, J. Chem. Soc. 175, 1936.
4. Sollmann, T., and Seifter, J.: Intravenous Injections of Soluble Bismuth Compounds: Their Toxicity, and Their Sojourn in the Blood and Organs, J. Pharmacol. & Exper. Therap. 74: 134, 1942.
5. Sollmann, T., Cole, H. N., and Henderson, K.: Clinical Excretion of Bismuth, Am. J. Syph., Gonor. & Ven. Dis. 22: 555, 1938.

LABORATORY METHODS

GENERAL

DEMONSTRATION OF TUBERCLE BACILLI IN TISSUE BY FLUORESCENCE MICROSCOPY*

GERMAIN CROSSMON, B.S., AND ERICH LOEWENSTEIN, PH.D., ROCHESTER, N. Y.

AT THE present time there is much interest in the identification of *Mycobacterium tuberculosis* in smears of pus, sputum, and body fluids by staining with a fluorescent basic dye, auramine O, and examination of preparations in ultraviolet illumination. The staining procedure is similar to the Ziehl-Neelsen method with the substitution of the fluorescent dye, auramine, for basic fuchsin. Thin smears are stained with carbolauramine usually at room temperature. The preparations are decolorized with acid alcohol, washed with water, and examined dry without a cover glass by means of the fluorescence microscope in ultraviolet light. Hagemann[1] in 1938 introduced this method. Since this publication, a number of papers[2, 3, 4, 5, 6, 7, 8, 9, 10] have been written confirming his results. These authors consider this method superior to the Ziehl-Neelsen technique for the detection of the tubercle bacillus in smears of sputum, pus, and body fluids for the following reasons:

1. The staining method with auramine is simpler than with basic fuchsin (Ziehl-Neelsen). Smears can be stained with carbolauramine at room temperature, while steaming carbolfuchsin is usually necessary for the Ziehl-Neelsen method.

2. Auramine, a di-phenyl methane basic dye, is probably a more specific stain than basic fuchsin for acid-fast organisms. (The number of tubercle bacilli stainable with carbolauramine appears to be greater than with carbolfuchsin.)

3. More tubercle bacilli are detected in smears stained with carbolauramine than with carbolfuchsin. Self-luminous, yellow fluorescent tubercle bacilli stained with the fluorescent dye, auramine, are more easily seen against a dark background than red-stained bacilli on a blue background. Bogen[6] reports in 1,000 duplicate smears stained in parallel by the Ziehl-Neelsen and fluorescent methods, there were over 20 per cent more positive tests by the newer technique.

*From the Medical Department and Scientific Bureau of Bausch & Lomb Optical Co.
Received for publication, December 3, 1942.

Lind and Shaughnessy[9] in an examination of 1,123 smears by means of the Ziehl-Neelsen and fluorescence methods found 106 to be positive. Twelve of these preparations, positive by the fluorescence method, were negative by the Ziehl-Neelsen method. They also state that because of the larger areas covered and ease of examination, the time required was reduced to one-fourth or one-third that required for the Ziehl-Neelsen method.

4. Smears are examined for tubercle bacilli in much less time. An oil immersion objective with a 10X eyepiece provides the usual magnification (930X) for examination of smears stained by the Ziehl-Neelsen method. Tubercle bacilli stained with auramine appear as yellow, fluorescent bodies on a dark field. The fact that the bacilli become secondary light sources serves to increase the contrast. Therefore lower magnification may be used, and thus a larger field may be examined at one time.

The fluorescence staining method as used for smears of sputum, pus, and body fluids may be modified for the detection of *Mycobacterium tuberculosis* in fixed tissue. The staining technique, equipment, and method of examination are as follows:

STAINING METHOD

1. Fix tissue in 10 per cent neutral formalin for twelve to twenty-four hours. Commercial formalin may be neutralized by the addition of marble chips a few days prior to fixation.

2. Dehydrate completely, imbed in paraffin and cut sections on a microtome at 6 to 10 microns. After removal of paraffin with xylol and passage through alcohol to water, place sections in carbolauramine. Since paraffin will fluoresce, it is essential that it be entirely removed by xylol.

Auramine O (National Aniline)	0.1 Gm.
Distilled water	100 c.c.
Liquefied phenol U.S.P.	4 c.c.

The auramine O is dissolved in the distilled water by shaking strongly in a flask. Gentle heating will hasten its solution. When the auramine is dissolved, the liquid phenol is added.

The application of heat shortens the staining time and usually increases the intensity of the stain. The following staining times are suggested:

Room temperature	30 minutes
Incubator 37° C.	15 minutes
Paraffin oven 55° C.	10 minutes
Gentle steaming of slides flooded with carbolauramine	3 to 5 minutes

4. Wash the preparations briefly in several changes of distilled water.

5. Decolorize in 95 per cent ethyl alcohol containing 0.5 to 1 per cent hydrochloric acid until the preparations are quite colorless. The time for decolorization does not usually require more than forty-five seconds. Two to three changes of acid alcohol should be used for this decolorization.

6. Wash preparations in distilled water.

7. Transfer to 0.1 per cent aqueous methylene blue, certification No. 18 (National Aniline), C. I. No. 922. Sections are stained from thirty seconds to one minute. As with the Ziehl-Neelsen method, the methylene blue removes any carbolauramine not removed by the acid alcohol with little sacrifice of the intensity of the auramine in the tubercle bacilli. In addition, by staining the tissue the methylene blue masks the natural fluorescence of the tissue and forms a dark background for the yellow, luminous tubercle bacilli.

8. Rinse in distilled water.

9. Dehydrate, clear in xylol, and mount in xylol-clarite. Most mounting media, including balsam, fluoresce in ultraviolet light. Tissue preparations are usually examined dry without a cover glass or mounted in nonfluorescing media such as distilled water, glycerin, or medicinal mineral oil. Although xylol-clarite fluoresces slightly, preparations mounted in this medium are permanent. In addition, its refractive index 1.515 is more favorable for the examination of stained elements.

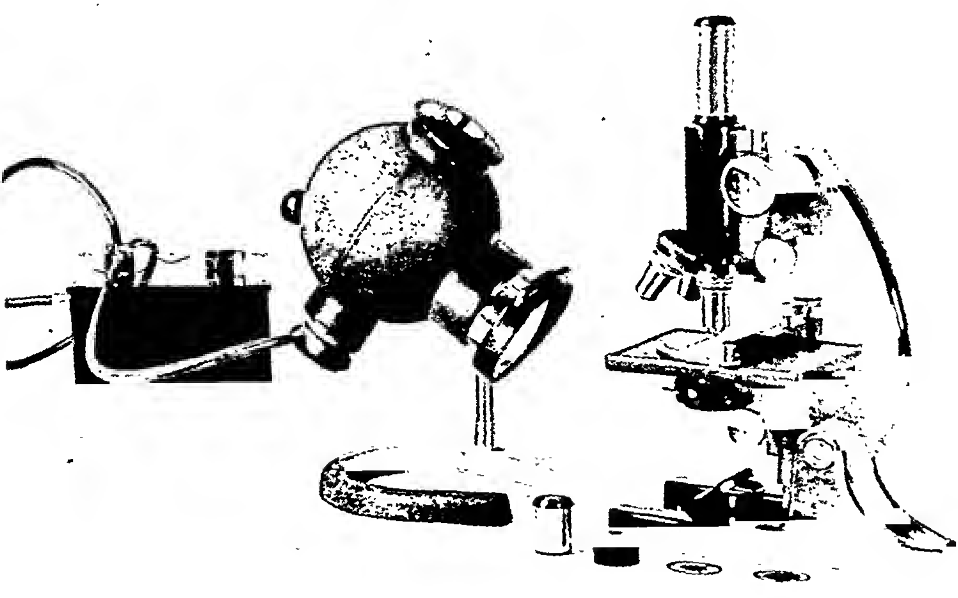


Fig. 1.—Equipment for fluorescence microscopy. Mercury vapor lamp, ultraviolet transmitting filter, Abbe Condenser 1.25 N.A., dark field stop 15 mm. diameter.

Any microscope (see Fig. 1) having a magnification of 430X and suitable for the examination of biologic material can be used for this work. The standard medical microscope is suggested. Although not absolutely necessary, the ordinary silvered glass mirror should be replaced with an aluminized mirror, for both glass and silver absorb ultraviolet.

As a source of illumination a mercury vapor lamp with iris diaphragm and filter holder is suggested. An ultraviolet transmitting glass filter and a dark field stop complete the equipment necessary for examination.

METHOD OF EXAMINATION

The preparations are best examined by enclosing three sides of the microscope with a shield to exclude extraneous light. A drop of nonfluorescent sandalwood oil or Shillaber's Immersion Oil is placed between the slide and condenser. The beam of light from the mercury vapor lamp is centered on the microscope mirror, and the condenser is focused up and down until a uniform distribution of light is obtained in the microscope field. The best condition is obtained when the substage condenser forms an image of the front aperture of the lamp in the plane of the specimen. A dark field stop is placed in the slotted ring below the substage condenser. Low power magnification with 5X or 10X eyepiece and 16 mm. objective is usually employed for the initial exploration. The observer should search for any luminous yellow bodies on a

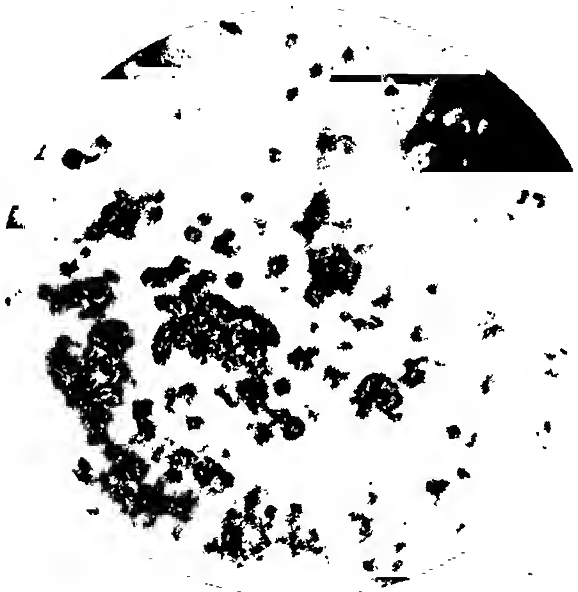


Fig. 2.—Photomicrograph of tubercle bacilli in lung tissue. Apochromatic objective 8.3 mm., 0.65 N.A. Eyepiece 10X Hyperplane. Magnification 400X. Exposure time: 7½ minutes.

dark field. Provided the staining technique has been properly employed, the only luminous structures in the field will be tubercle bacilli. A higher magnification, 5X or 10X eyepiece with 4 mm. objective, should now be used to inspect the morphology more closely. Tubercle bacilli appear as thin, yellow, luminous, slightly curved rods on a dark background. (See Figs. 2 and 3.)

Variations in the method of examination will be suggested to the observer. For example, one may prefer to use an 8 mm. objective with a 20X ocular, or for higher magnifications, a 15X or 20X eyepiece with the 16 mm. or 4 mm. objective. Although an oil immersion objective is not necessary, it may be used even in an undarkened room with a 5X or 10X ocular using Shillaber's immersion oil, sandalwood oil, or synthetic oil of wintergreen between the slide and objective. If desired, preparations may be first focused and examined in visible

light by using a daylight filter or a ground glass and blue filter. This will show the nuclei of the tissue cells and other basophilic elements that may be present, stained blue by the methylene blue. In the case of smears of sputum, pus, and body fluids, the blue staining of the nuclei of leucocytes can be observed. If many tubercle bacilli in the preparation are concentrated at one spot, it is even possible at this time by partially closing the diaphragm and using a high dry 4 mm. objective to locate this auramine-stained area in visible light. It appears as a pale yellow area on the methylene blue background. According to Gage,[11] a long card smeared with anthracene in a strong solution of cane sugar and dried makes a good detector for focusing the beam upon the aluminized mirror, and a similar preparation on a corex glass slip serves well for making sure that the ultraviolet is passing up through the condenser to the place where the preparation is to be located. A piece of fluorescent glass or cellophane might be used just as well for the same purpose.

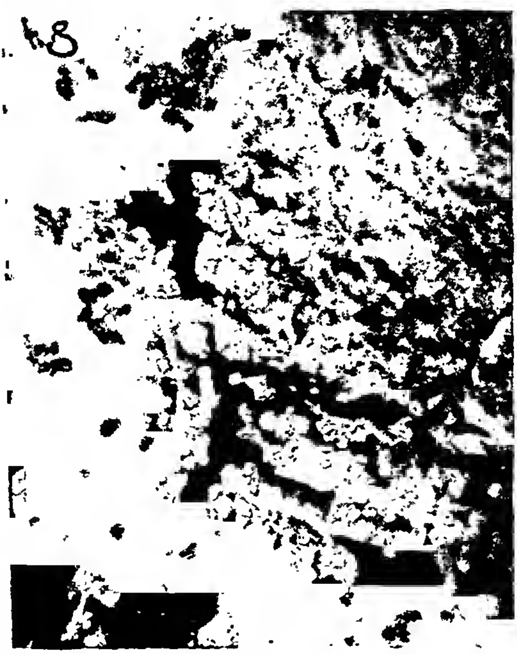


Fig. 3.—Photomicrograph of tubercle bacilli in lung tissue. Achromatic objective 4 mm., 0.65 N.A. Eyepiece 7½ Hyperplane. Magnification 400×. Exposure time: 30 minutes.

Distilled water or glycerin does not fluoresce, and although their refractive index is not that of immersion oil, they can be used in place of sandalwood oil or Shillaber's Immersion oil to make contact between the slide and condenser.

SUMMARY

A staining method and equipment* for the detection of tubercle bacilli in tissue is described. Tissue sections 6 to 10 microns are stained with carbol-auramine solution and decolorized with acid alcohol. Preparations are counterstained with methylene blue, dehydrated, cleared, and mounted in xylol-clarite

*The equipment, including mercury vapor lamp, dark field stop, filter, metal shield, and aluminized mirror may be obtained from Bausch & Lomb Optical Co., Rochester, N. Y.

for permanent preparations. Distilled water, glycerin, medicinal mineral oil, or Shillaber's Immersion oil may also be used as mounting medium, provided the cover glass is sealed with shellac or paraffin wax. The preparations are examined with ultraviolet radiation using low power magnification (50X or 100X) and a dark field stop for the initial examination. Higher magnification (430X) is necessary to check the morphology of stained elements. Tubercle bacilli appear as thin, yellow, luminous, slightly curved rods on a dark background.

The staining method and fluorescence microscope have also proved satisfactory for the detection of *Mycobacterium tuberculosis* in sputum, pus, and body fluids. One of us, Erich Loewenstein, has successfully used this method for the detection of lepra bacilli in smears.

We acknowledge with thanks the assistance of Mr. J. V. Butterfield of the Scientific Bureau of Bausch & Lomb for making the photomicrographs shown in this paper.

REFERENCES

1. Hagemann, P. K. H.: Fluoreszenzfärbung von Tuberkelbakterien mit Auramin, München. med. Wchnschr. 85: 1066, 1938.
2. Herrmann, W.: Der Nachweis von Tuberkelbazillen mit dem Fluoreszenzmikroskop, Deutsche med. Wchnschr. 64: 1351, 1938.
3. Keller, C. J.: Vereinfachter Nachweis von Tuberkelbazillen im Fluoreszenzlicht, München. med. Wchnschr. 85: 2024, 1938.
4. Didion, H.: Über den fluorescenzmikroskopischen Nachweis von Tuberkelbakterien, Klin. Wchnschr. 18: 1315, 1939.
5. Küster, H.: Untersuchungen mit dem Fluoreszenzmikroskop, Deutsche med. Wchnschr. 65: 92, 1939.
6. Richards, O. W., and Miller, D. K.: An Efficient Method for the Identification of M. Tuberculosis With a Simple Fluorescence Microscope, Am. J. Clin. Path., Tech. Supp. 5: 1, 1941.
7. Richards, O. W., Kline, E. K., and Leach, R. E.: Demonstration of Tubercle Bacilli by Fluorescence Microscopy, Am. Rev. Tuberc. 44: 255, 1941.
8. Bogen, E.: Detection of Tubercle Bacilli by Fluorescence Microscopy, Am. Rev. Tuberc. 44: 267, 1941.
9. Lind, H. E., and Shaughnessy, H. J.: Fluorescent Staining Technique for Detection of Acid-Fast Bacilli, J. Lab. & Clin. Med. 27: 531, 1942.
10. Graham, C. F.: Simplification of Fluorescence Microscopy, J. Lab. & Clin. Med. 27: 1188, 1942.
11. Gage, S. H.: The Microscope, ed. 15, Ithaca, N. Y., 1932, Comstock Pub. Co., p. 244.

AN IMPROVEMENT OF THE SEROLOGIC KAHN REACTION IN THE SPINAL FLUID*

F. RAPPAPORT, PH.D., AND D. RAPPAPORT, PH.D., PETAH TIQVA, PALESTINE

THE Kahn test is carried out with ease and gives very reliable results in serum. In spinal fluid, however, various difficulties arise, so that other methods have been preferred. These difficulties result mainly from the attempt to concentrate the effective antibodies through precipitation with ammonium sulfate, as recommended by Kahn himself. In this method, the spinal fluid is mixed with an equal quantity of a saturated solution of ammonium sulfate and well shaken, thus precipitating the globulins. Inactivation is obtained by heating the mixture at 56° C. for twenty minutes. It is then centrifugated at high speed and the supernatant fluid is poured off.

The following factors may lead to inaccuracies:

1. A certain quantity of antibodies may be dissolved in the supernatant sulfate solution, and when the latter is poured off, they are lost for the reaction.

2. The spinal fluid may be poor in globulins, so that, although it may be rich in antibodies, only a fraction of them is precipitated together with the present amount of globulins.

3. The salt concentration should always be the same and should be reproduced exactly. The considerable quantity of ammonium sulfate which may remain in the precipitate obviously changes the salt concentration in which the reaction is going to take place.

4. Finally, the scarcity of albumin in the spinal fluid may lead to a pseudopositive reaction, for the flocks of the antigen which are normally dissolved in serum may remain suspended in a fluid poor in albumin.

Considering that the inaccuracies mainly arise from the attempt to concentrate the antibodies in the spinal fluid, we developed a method for spinal fluid, similar to the one used for serum.

Reagents employed:

I. a. Kahn antigen
   b. 0.9 per cent NaCl solution
II. A reliable nonluetic serum (Kahn negative)

The antigen is thoroughly mixed with the salt solution to the given titer and left to ripen for at least ten minutes at room temperature.

PROCEDURE

Pour 0.1 c.c. of serum (reagent II) in each of two small test tubes and inactivate it at 56° C. for twenty minutes, together with a little more than

*From the Laboratory of the Beilinson Hospital, Petah Tiqva, Palestine.
Received for publication, August 3, 1942.

1.5 c.c. of saline solution. Remove tubes from water bath and cool to room temperature. Add 0.01 c.c. of the ripened antigen (I) to each of the serum tubes and shake by hand or shaker for three minutes. Add 0.5 c.c. of spinal fluid to one tube and 1.0 c.c. to the other, and again shake for three minutes. The reaction is now read in the usual way. If a precipitate has formed only in the tube containing 1.0 c.c. of spinal fluid, the reaction is weakly positive; if in both tubes, it is considered strongly positive. If only small quantities of spinal fluid are at disposal, the test may be made with one tube and 0.75 c.c. of spinal fluid. To make the reaction more clearly visible, it is advisable to centrifuge at a high speed for about ten minutes. If the reaction is positive, a precipitate on the bottom of the test tube is seen, the supernatant fluid being transparent. After shaking the test tube, the flocculation is distinctly visible. In case of a negative reaction the fluid remains turbid and there is no precipitate. For measuring accurately the small amount of antigen, we use a 0.200 c.c. serologic pipette with a bent tip (Fig. 1), which enables us to direct the fluid to one spot. By using this pipette, dispersion and loss of substance on the wall of the test tube are prevented.

In order to prove that the spinal fluid itself does not affect or modify the reaction, we added to 4 test tubes containing 0.1 c.c. of inactivated Kahn positive serum, the usual quantity of antigen. After shaking the tubes, we added to two, 0.5 c.c. and 1.0 c.c. of negative spinal fluid, and to the other, two equal quantities of salt solution. The reaction in both cases was the same in character and in degree. Negative sera treated with negative spinal fluid never gave positive results.

A serologic reaction for gonococcus and echinococcus antibodies, based on the same principle, will be reported shortly.

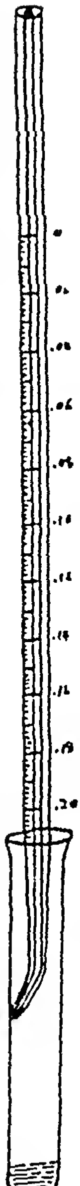


Fig. 1.

AN EFFECTIVE METHOD FOR ANESTHETIZING SMALL ANIMALS*

Harold M. Kaplan, Ph.D., Cambridge, Mass.

IN THE laboratory investigation of clinical problems in which small animals are utilized, the problems of struggling and restraint are present. The difficulties arise particularly in carrying out ether anesthesia without adequate assistance upon animals such as the cat.

One can readily work alone without difficulty by placing not too docile animals within a wooden box. If the dimensions of length, height, and width, respectively, are about 16 × 13 × 11 inches, it adequately accommodates any cat or smaller animal. Windows should be inserted in at least two of the sides so that the animal's condition is observable. A hinged cover is constructed on top of the box.

An ether vaporizer to be used with the box has been devised here. This is illustrated in Fig. 1. It is constructed quickly and easily with little expense, and its parts are obtainable.

The ether is contained in a closed separatory funnel, and is allowed to run through the stopcock into the vaporizing flask when needed. The flask must be Pyrex and widemouthed, a convenient capacity being 500 c.c. It must be fitted with a rubber stopper. Erlenmeyer flasks of this capacity, which take a No. 10 stopper, are manufactured by Corning Glass Works, Corning, N. Y. An eight-ounce widemouthed nursing bottle with a No. 10 stopper may be substituted, but it will not take as much ether. Two wires are each inserted into a glass tube, and the tube at its two ends is flame-sealed around the wire. These shielded wires are inserted through the stopper to extend down into the flask. Near the bottom, each wire leaves the glass shield and is firmly soldered or welded to a broad flat copper electrode; the two electrodes are kept carefully separated to prevent sparking (which might explode the ether). Above the flask, the wires are extended upward by well-insulated cord through a switch to a plug. This may be inserted into any socket carrying alternating current (110 to 220 volts). The switch must be well-enclosed, by tape or other means, to prevent the chance of open sparking.

An aqueous copper sulfate solution is poured inside the flask. Its level must be kept well *above* the electrodes. If the solution is not too concentrated (a pale blue color indicates the proper saturation), its electrical conductivity is small; and thus when the current is applied, its resistance will create heat. This rapidly warms the ether to its boiling point (about 35° C.), and vaporizes it. The vapor is conducted away from the flask in a steady stream through glass and rubber pressure tubing to the animal box. A terminal glass nozzle should penetrate the box through a fitted hole bored at a low level to allow the ether vapors to rise and permeate the whole interior.

*From the Department of Physiology, Middlesex University, Waltham, Mass.
Received for publication, October 19, 1942.

Within the vaporizing flask the volume of ether is always observable, since being light and immiscible with water it rests as a distinct layer above the blue solution. There is no danger of its diffusing downward to the electrode area. Sparking between the electrodes is further minimized by using an alternating current. This alternation also prevents appreciable solution of copper, which would erode the electrodes and make the fluid muddy.

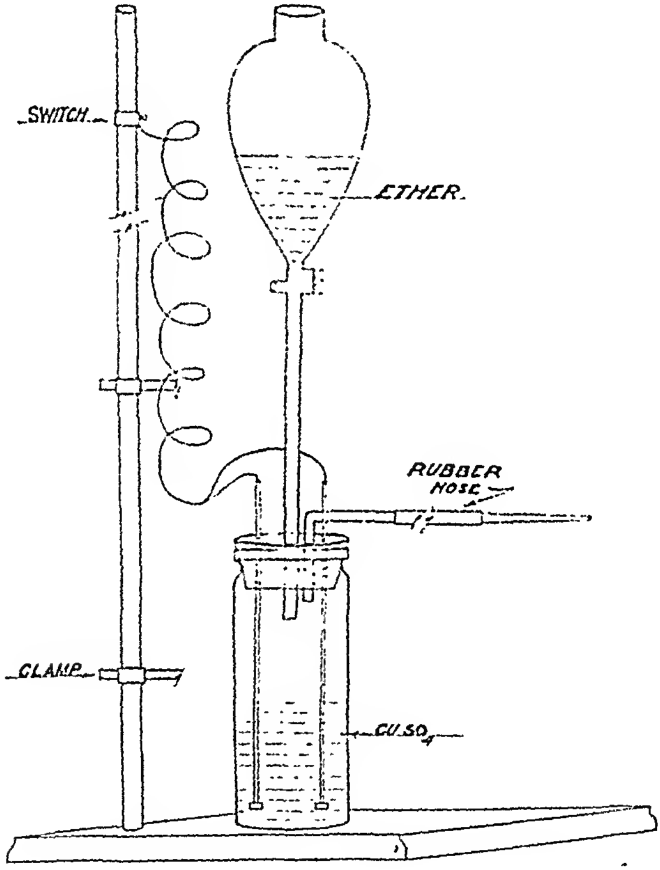


Fig. 1.

We use this apparatus frequently and can state that with ordinary care it is no more dangerous than any other laboratory appliance, although it is unquestionably hazardous in the hands of inexperienced persons. It is quick and sparing of ether. By observation of the ether box, the stages of anesthesia are clearly followed. In the surgical stage the animal may be removed and tied down to an animal board. It is kept anesthetized thereafter by applying a cone to its nose.

CHEMICAL

SERUM BILIRUBIN: A PROCEDURE FOR THE DETERMINATION OF INDIRECT AND DIRECT VALUES

BERNARDO SEPULVEDA, M.D.,* AND ARNOLD E. OSTERBERG, PH.D.†
ROCHESTER, MINN.

THE original van den Bergh technique and the modification to this method introduced by Thannhauser and Andersen have been used extensively for the determination of serum bilirubin. Both procedures, however, present several disadvantages which can be summarized as follows: (1) It is difficult to match the color of azorubin with that of artificial standard solutions; (2) loss of bilirubin occurs in the protein precipitate; (3) values of less than 1 mg. cannot be read accurately; (4) separate determinations of the direct and indirect fractions cannot be made since estimation is on total bilirubin; and (5) in the Thannhauser and Andersen method the personal error involved in the correct differentiation of direct and indirect types of reaction is considerable.

The fact that sera in which the bilirubin gives a direct reaction also contain a varying amount of indirect reacting bilirubin is theoretically of interest. It has been postulated that the proportion between these two types of bilirubins is different in various types of jaundice, and therefore their separate determination should give diagnostic and prognostic information. Consequently, several techniques have been devised to measure separately the quantity of direct and indirect fractions of total bilirubin; furthermore, new techniques have been devised which avoid the deficiencies of the former methods and provide for the accurate determination of bilirubin. In this paper a technique for determination of bilirubin is described which is based in part on two methods previously outlined.

EARLIER METHODS FOR MEASURING BILIRUBIN

Varela Fuentes[1,2] developed a technique on the basis of the solubility of indirect reacting bilirubin in chloroform. In his procedure the indirect reacting bilirubin. Therefore we subtract from the final reading of direct bilirubin this After evaporation of the chloroform the dry residue is redissolved in chloroform and alcohol and then diazotized. The concentration of direct bilirubin in the same sample of serum is determined by adding diazo reagent and alcohol after extraction of the indirect bilirubin with chloroform. The precipitate is separated by filtration, and the reading is made on the filtrate by means of a standard colorimeter.

The technique for the extraction of indirect reacting bilirubin proposed by Varela Fuentes appeared to us to be the most efficient method thus far reported.

*Fellowship in Medicine, Mayo Foundation.
†From the Section on Clinical Biochemistry, Mayo Clinic, Rochester, Minnesota.
We wish to express our appreciation to Miss Elizabeth Machy for technical assistance.
Received for publication, March 5, 1943.

It has been used in the work to be described in this paper with slight modifications which were made to adapt it to the photelometer.

Several objections can be raised to the use of the method of Varela Fuentes for the determination of direct bilirubin; essentially it has the same disadvantages as the original van den Bergh method and the Thannhauser and Andersen technique, that is, loss of bilirubin in the protein precipitate and the difficulties of matching color with that of artificial standard solution.

One of us (A. E. O.)[3] modified the technique for determination of bilirubin proposed by van den Bergh and Grotepass[4] in order to adapt it for reading in the photelometer. Their procedure for determination of direct reacting bilirubin is based on the fact, known since the first publications of van den Bergh, that this fraction couples immediately with the *diazo* reagent in the serum; the addition of 96 per cent alcohol is unnecessary for the reaction. In their technique 50 per cent buffer alcohol (final concentration in tube, 25 per cent) is used to maintain a constant pH in the tubes for the direct and indirect reaction. The use of alcohol, however, even in such a low concentration, is a cause of error, for it allows part of the indirect bilirubin to react and thus increases the color of the direct reaction. This fact has been mentioned previously by Malloy and Evelyn[5] and has been confirmed in the present investigation. The buffer alcohol is not necessary when the photelometer is used, since the differences in pH do not exert any influence on the reading. In the determination of indirect bilirubin the method obviously can be applied only in those cases in which the serum contains this fraction alone, as in certain cases of hemolytic icterus. When both fractions are present, as usually occurs in obstructive and parenchymatous jaundice, the method really determines total bilirubin. Even so, the estimation is incomplete, since as mentioned before some direct bilirubin is carried out in the protein precipitate.

Malloy and Evelyn developed a procedure to make separate determinations of direct and total bilirubin. A considerable improvement is made by the use of 50 per cent alcohol in the measurement of total bilirubin; Malloy and Evelyn have demonstrated that complete coupling of the bilirubin takes place in the presence of 50 per cent alcohol without any precipitation of protein. The estimation of direct bilirubin is based on the same principle we have used, namely, the ability of this fraction to react in water solution; the reading is also made in a photelometer. Later some differences between the two methods will be mentioned.

DESCRIPTION OF PRESENT TECHNIQUE

Qualitative Test.—The qualitative test is first performed on a sample of serum as follows. To 1 c.c. of serum, generally diluted, 2 c.c. of diazo reagent is added. Three well-known results may be obtained:

1. A direct reaction may occur. The color change then is detected as soon as serum and diazo reagent are mixed together; it reaches its maximal intensity in from sixty to ninety seconds. The color is pink in undiluted serum and purplish in diluted serum.

2. An indirect (delayed or negative direct) reaction may occur. No change of color then is noted during the first two minutes after addition of the diazo

reagent. Between four and ten minutes after the diazo reagent is added a golden color appears, in about thirty minutes a brownish color, and after one to one and a half hours the characteristic pink color develops. This color is similar to that of the direct reaction in the undiluted serum.

3. A biphasic, direct reaction may occur. This type of reaction has been found only three times in more than 400 cases observed by us. The color which appears during the first minute is not a typical pink, but a brownish red which generally reaches its maximal intensity in two or three minutes after the diazo reagent is added.

Quantitative Test.—After the qualitative test, quantitative determination of direct and indirect reacting bilirubins is made.

Direct Reacting Bilirubin.—The quantitative determination of direct reacting bilirubin is carried out only if either a direct or biphasic reaction has occurred in the qualitative test. The volume of the serum and diazo reagent is made up to 10 c.c. with distilled water, and the photelometer reading is made during the first ten minutes after the water is added.

In spite of the fact that a filter having a maximal transmission at 530 to 535 millimicrons is used, the color of the serum itself influences the reading. By making a control series of readings with normal sera against water "blank," we have found an average photelometer reading of 96 which equals 0.4 mg. of bilirubin. Therefore we subtract from the final reading of direct bilirubin this correction factor of 0.4 mg. This procedure is accurate and avoids the preparation of a normal "serum blank" for every determination.

Indirect Reacting Bilirubin.—To another 1 c.c. portion of the serum in a centrifuge tube marked at 10 c.c. is added 2 c.c. of sodium sulfate (12 per cent solution prepared from anhydride), and the solution is mixed. To this is added 5 c.c. (or more) of chloroform. The tube is shaken vigorously for thirty seconds, then centrifuged for about five minutes at high speed. The yellow chloroform extract remains under the turbid serum. The capillary end of a pipet is introduced to the bottom of the tube and almost all the chloroform is aspirated. A slanting mirror should be placed under the tube so the operation may be watched. The aspirated chloroform is transferred to a test tube of at least 2 cm. in diameter. Extraction of the serum is repeated with a new portion of chloroform and the second extract is added to the first. Extraction must be repeated until the chloroform is colorless. Usually three to four extractions are sufficient.

The combined chloroform extracts in the test tube are evaporated in a water bath at 80° C. The dry adherent residue is taken up in 0.5 c.c. of chloroform and 2 c.c. of 96 per cent alcohol, which dissolve the bilirubin. To this is added 0.5 c.c. of a fresh diazo reagent (final volume 3 c.c.). The red color of azobilirubin does not develop completely until after ten minutes. The reading is made in the photelometer against water blank.

We have used the Cenco Sheard-Sanford photelometer equipped with a Cenco No. 2 filter which has a transmission range of 550 to 700 millimicrons. The calibration curve is obtained by the following procedure: Five milligrams of purified bilirubin is dissolved in 100 c.c. of chloroform, and 10 c.c. of this solution is evaporated under carbon dioxide on a water bath, until it is nearly dry. To the residue is added 80 c.c. of a solution consisting of 50 per cent alcohol which contains 10 c.c. of a buffer solution of pH 6.6 to every 100 c.c. The buffer alcohol solution is heated for some time in order to evaporate the chloro-

form. Next, the solution is brought to room temperature and to it is added 20 c.c. of the reagent consisting of Solution A, 1 Gm. sulfanilic acid, 15 c.c. of 25 per cent hydrochloric acid with distilled water up to 1 liter, and Solution B, 0.5 per cent sodium nitrite in aqueous solution. Ten cubic centimeters of Solution A is mixed with 0.3 c.c. of Solution B shortly before use. Additional buffer containing alcohol is added to the solution after the reagent and the total quantity has been brought up to 100 c.c. The pigmented solution which is to be examined must be left in the dark for fifteen minutes in order to complete the coupling.

The concentration of bilirubin as its azo derivative equals *1/200,000* or *0.5* mg. in 100 c.c. This corresponds to a concentration of bilirubin of 5 mg. in each 100 c.c. of serum when the analysis is carried out on 1 c.c. of serum and the final volume equals 10 c.c.

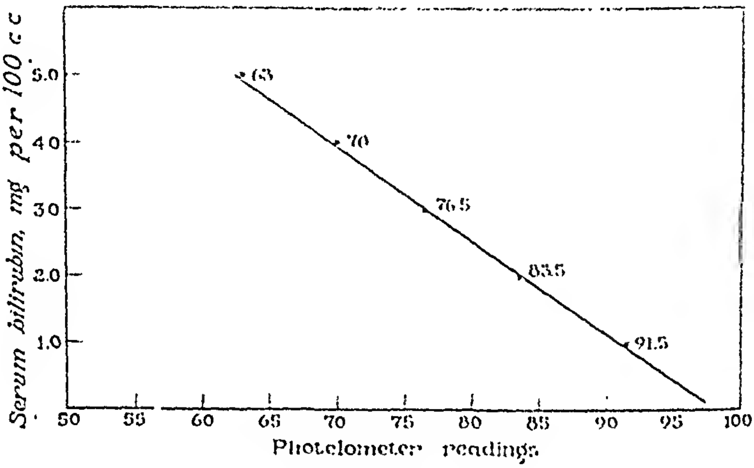


Fig. 1.—Curve of photelometer readings showing relationship with concentration of serum bilirubin.

Other points on the curve are obtained in a similar manner using volumes of the original solution of bilirubin of 8 c.c., 6 c.c., 4 c.c., and 2 c.c. By this procedure, as is shown in Fig. 1, there is obtained, for practical purposes, a straight line relationship* up to values corresponding to a concentration of 3 mg. of bilirubin in each 100 c.c. of serum. If higher concentrations than this value are obtained, it is necessary to dilute the serum previous to the determination in order to obtain a value of less than 3 mg. in each 100 c.c. Appropriate factors are then applied to obtain the final value of bilirubin. This procedure is adopted because of the fact that when values of more than 3 *mg. per* 100 c.c. are subjected to the diazo reaction, the photelometric readings depart too greatly from the assumed straight line relationship. Also, the concentration of the compound produces a dark color and turbidity may develop.

COMMENT

Direct Reacting Bilirubin.—We have found that in serum diluted before the addition of diazo reagent the reading of direct bilirubin yields higher figures

*It is known that, in the case of solutions of substances which possess characteristic absorption zones, the concentration (C) is proportional to the negative logarithm of the transmitted light (I); that is, $C = -k \log I$. For the range of dilutions used in these investigations, however, departures from the linear relationship as shown in Fig. 1 and the values obtained from the semilogarithmic plot are not considered as being significant.

than in undiluted serum. Serum diluted after coupling behaves like undiluted serum. The higher the concentration of pigment, the greater the difference in the results. A curve of the quantities obtained at different dilutions can be drawn demonstrating that relationship, until a limit of dilution is reached, after which no further variation occurs (Table I).

These differences were first attributed to variations in the color of the reaction, owing to changes in pH, the azorubin in undiluted serum being red-pink and purplish or red-violet in diluted serum. In the first case the proteins and other buffer mechanisms present in serum neutralize partially the acid diazo reagent giving a final reaction that is weakly acid. In the diluted serum this does not occur and a lower pH results. Griffiths and Kaye[6] have mentioned the diverse tones of color present according to the variations in pH. In the experiment described in Table I, however, we found that these differences in color did not exert an influence on the reading; also that the final results were comparable when several different fluids were used to make the dilutions.

TABLE I

READINGS ON SERUM DILUTED BEFORE AND AFTER COUPLING

	BILIRUBIN, MG. PER 100 C.C. AFTER USE OF DILUTIONS OF		
	1:10	1:20	1:40*
A. Serum diluted after coupling	22.6	24.0	24.0
B. Serum diluted before coupling			
Dilution with serum of normal dog†	26.6	32.6	37.6
Dilution with diazo reagent	24.6	27.6	31.6
Dilution with water	27.6	31.6	35.6

*Dilutions greater than 1:40 gave same results.
†The serum of a normal dog contained 0.05 mg. per 100 c.c. of indirect bilirubin.

The higher readings obtained in serum diluted before coupling may be explained as follows: When the concentration of direct bilirubin is high, the diazo reagent is unable to cause all the bilirubin to react; consequently a variable amount remains undetermined. In diluted serum less pigment is present, and with the same quantity of diazo reagent the combination is complete and the total quantity of direct bilirubin present is determined. The proper dilution for serum in a case of jaundice can be judged with relative certainty by the intensity of the yellow color of the serum. We usually make a 1:5 or 1:10 dilution. After diazotization has taken place, the reading is made in the photelometer; if the reading is below the equivalent of 3 mg. (above 75 on the scale), the dilution is correct and the calculation can be made therefrom in milligrams of bilirubin. When the reading is higher than 3 mg. (below 75 on the scale), the dilution is increased. With diluted serum we have found that the most accurate results are obtained if the readings are kept between 1 and 3 mg. (75 to 90 on the scale). The disadvantage of repetition of the reading is necessary only in case of unknown sera and with practice it can usually be avoided.

We do the reading of direct bilirubin the first ten minutes after the water is added, because after this time the indirect bilirubin also takes part in the reaction. It is well known that the indirect bilirubin can react slowly in water solution, and this is evidenced by the fact that in sera in cases of hemolytic jaundice the development of color starts usually after ten minutes. When both

fractions are present, we have observed differences of several milligrams between the readings at five and thirty minutes. This is especially true when the amount of indirect bilirubin is high.

Indirect Reacting Bilirubin.—Grunenberg[7] first noted the different behavior with chloroform of direct and indirect reacting bilirubins and demonstrated that the pigment in serum from a patient who had hemolytic jaundice was soluble in chloroform while that from a patient who had obstructive jaundice was not. Andrewes,[8] Collinson and Fowweather,[9] Roberts[10] and Newman[11] have confirmed these results. Hunter[12] included the different solubilities in chloroform in a summary of the properties of direct and indirect reacting bilirubins.

De Castro[13] presented a method for the determination of both types of bilirubin in which the indirect type of bilirubin was extracted from the serum with chloroform. Varela Fuentes showed that with this technique, when 2 c.c. of chloroform were used and the extraction was done only once, 50 per cent of the indirect reacting bilirubin remained in the serum. Consequently he modified the method so that 5 c.c. of chloroform were used and several extractions were made. In most cases extraction by this method was complete, but when the concentration of bilirubin was high, a small portion of the pigment remained in the serum. With the use of the 12 per cent solution of sodium sulfate, extraction was improved and the chloroform removed practically all the indirect bilirubin.

Before adopting the technique of Varela Fuentes it was necessary for us to confirm that: (1) chloroform extracts only indirect reacting bilirubin without affecting the direct type, and (2) chloroform extracts the indirect reacting bilirubin completely. This was done as follows:

In serum in which both direct and indirect reacting bilirubins were present, the direct bilirubin was measured by the method recommended in this paper. In a different sample of the same serum complete extraction of the indirect bilirubin with chloroform was carried out. After extraction, a mixture of serum, sodium sulfate, and chloroform residues remained in the tube. After centrifuging, the three layers separated: the upper layer was a yellow liquid (mostly sodium sulfate in which was dissolved part of the direct bilirubin); the middle layer was the protein precipitate which carried the remainder of the direct reacting bilirubin, and the bottom layer consisted of a small quantity of colorless chloroform. The upper layer was poured into another tube and the chloroform was evaporated in the presence of carbon dioxide to prevent oxidation of the bilirubin. The dry precipitate was dissolved in 10 to 20 c.c. of 5 per cent urea solution. Usually all the precipitate went into solution but sometimes a portion remained insoluble. To this solution was added the yellow upper layer mentioned and 1 c.c. of the mixture was diazotized. A direct reaction was obtained and the reading was made in the photelometer. In a good proportion of cases approximate agreement was found between the values for direct bilirubin and those of bilirubin recovered from serum after extraction with chloroform (Table II). Cases in which recovery of direct bilirubin was not complete were those in which a portion of the precipitate containing some bilirubin was insoluble. Apparently this fact accounts for the incomplete recovery of direct reacting bilirubin in these cases.

It is easy to observe that after several extractions the original yellow serum of hemolytic jaundice turns a whitish color. On the other hand, the serum of ob-

structive jaundice remains yellow no matter how many extractions are made with chloroform. A more definite proof of the complete extraction of bilirubin with chloroform was obtained by the following method: Three c.c. of 96 per cent alcohol were added to serum from a patient who had hemolytic jaundice after complete extraction with chloroform. The mixture was shaken and centrifuged. The supernatant fluid was pipetted off and diazotized. In a great majority of cases no color developed; its absence indicated the absence of bilirubin in the serum. We found that the only time at which a small portion was not extracted was in the presence of hemolytic jaundice, when the concentration of bilirubin in the serum was high. This portion was never more than 15 per cent of the whole amount of indirect reacting bilirubin present. Therefore, from a practical standpoint, it may be considered that chloroform produces a complete extraction of indirect reacting bilirubin. In this point we agree with the results reported by Varela Fuentes.

TABLE II

VALUES FOR DIRECT REACTING BILIRUBIN IN SERUM BEFORE AND AFTER EXTRACTION OF INDIRECT BILIRUBIN WITH CHLOROFORM

DIRECT BILIRUBIN		INDIRECT BILIRUBIN
BEFORE EXTRACTION	AFTER EXTRACTION	
13.8	14.4	3.9
14.1	13.2	2.5
44.6	43.7	3.7
27.5	28.0	2.7
16.8	17.0	2.7
22.6	23.0	2.4

TABLE III

COMPARISON BETWEEN NEW METHOD AND THE THANNHAUSER AND ANDERSEN METHOD IN HEMOLYTIC PROCESSES

DIAGNOSIS	NEW METHOD		THANNHAUSER AND ANDERSEN METHOD	
	DIRECT BILIRUBIN	INDIRECT BILIRUBIN, MG. PER 100 C.C.	REACTION	MG. PER 100 C.C.
Congenital hemolytic icterus	0	3.2	Indirect	2.6
Congenital hemolytic icterus	0	10.0	Direct	9.5
Congenital hemolytic icterus	0	2.5	Indirect	2.9
Congenital hemolytic icterus	0	1.3	Indirect	1.6
Acquired hemolytic icterus	0	2.8	Direct	4.1
Subacute hemolytic anemia	0	1.5	Direct	2.4
Acquired hemolytic anemia	0	5.4	Direct	5.8
Acute hemolytic icterus	0	9.6	Direct	10.7

COMPARISON OF NEW METHOD WITH OTHER METHODS

Thannhauser and Andersen Procedure.—Qualitative reaction.—The qualitative results obtained with the new technique and with the Thannhauser and Andersen technique in a group of cases of hemolytic processes are listed in Table III. In five cases the results with the Thannhauser and Andersen technique were incorrect. Apparently the short period of time, two minutes, between the addition of the diazo reagent and the reading of the qualitative test used in the Thannhauser and Andersen method accounts for the wrong reports. When the concentration of indirect bilirubin is high, a change of color starts at the

end of two minutes; this color is never the characteristic pink or red violet of direct bilirubin but may lead to misinterpretation.

Quantitative Reaction.—The quantitative results from the same two techniques are shown in Fig. 2. In every case the values of total bilirubin were higher with the new technique. The higher the quantity of bilirubin present the greater was the difference in the readings given by the two methods. Occasionally the concentration was found to be as much as three times as high with the new technique.

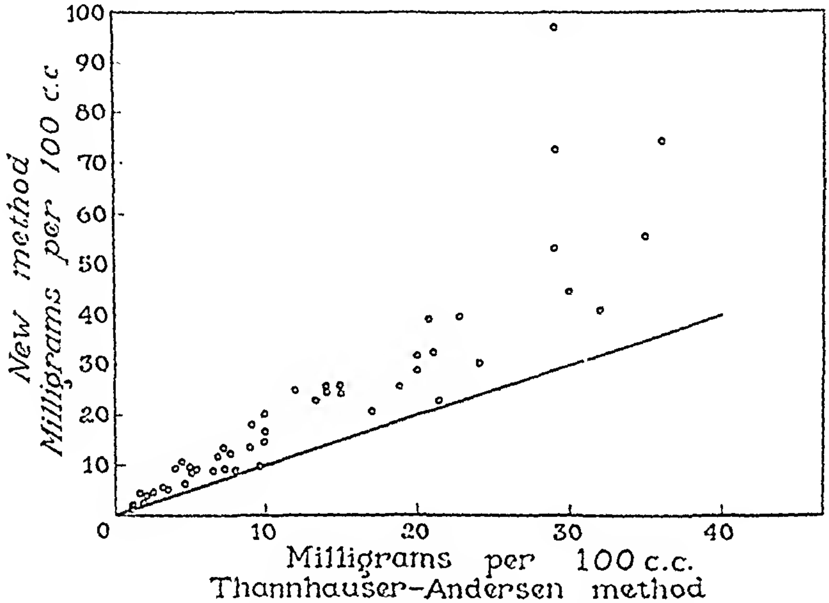


Fig. 2.—Determination of bilirubin in 65 cases of intrahepatic and obstructive jaundice. Quantitative results.

TABLE IV

COMPARISON OF MALLOY AND EVELYN TECHNIQUE AND THE NEW TECHNIQUE: QUANTITATIVE AND QUALITATIVE RESULTS

CASE	BILIRUBIN, MG. PER 100 C.C.				
	MALLOY-EVELYN TECHNIQUE		NEW TECHNIQUE		TOTAL
	DIRECT	TOTAL	DIRECT	INDIRECT	
1	1.0	2.5	Negative	2.5	—
2	0.3	0.8	Negative	0.3	—
3	0.7	1.2	Negative	0.8	—
4	1.2	1.5	1.5	0.3	1.8
5	5.0	7.4	6.5	0.2	6.7
6	10.0	13.5	13.6	1.1	14.7
7	7.5	10.4	10.6	1.0	11.6
8	0.7	1.7	2.6	0.6	3.2

Malloy and Evelyn Technique.—In Table IV the results of the new technique and the Malloy-Evelyn technique are compared. In the latter, determinations are made of the direct reacting bilirubin and of total bilirubin. Quantitative results for total bilirubin were similar, and from a practical standpoint both methods apparently are equally reliable. The qualitative results disagreed, however, because in several cases "direct" reacting bilirubin was found with

the Malloy-Evelyn technique whereas it was not found with the new technique. The explanation for this apparent contradiction is as follows: In the Malloy and Evelyn technique readings for direct bilirubin are always made regardless of the qualitative type of reaction. In the new technique, readings for direct bilirubin are made only when the qualitative reaction gives evidence of the presence of direct bilirubin. As has been stated, indirect bilirubin can react slowly in water solution; consequently, if a reading is made thirty minutes after the diazo reagent has been added to a serum from a normal person or to one from a patient who has hemolytic jaundice, the coupling is advanced enough to give a false direct reaction. This ability of the indirect bilirubin to react slowly in water solution explains, in our opinion, the results reported by Cantarow, Wirts, and Hollander,[14] who used the Malloy and Evelyn technique. These writers made the reading at the end of thirty minutes and found what they considered direct reacting bilirubin in the serum of normal persons, in cases of hemolytic jaundice, and in cases of hepatic and biliary disturbances; the values for total bilirubin, however, were within normal limits. Furthermore, they found that in some cases of hepatic and biliary disease the qualitative test gave a negative direct result while the quantitative procedure indicated the presence of "direct" bilirubin. This apparent contradiction is due to the fact that in the period of time which elapsed before the reading was made some of the indirect bilirubin was changed to azorubin. It is true that those results can be taken as a conventional term and probably the values would be comparable in the same group of disorders. We think it preferable, however, to establish a clear distinction between the qualitative reactions and evaluate the direct bilirubin only when its presence has been proved by an immediate reaction.

SUMMARY

A technique for the determination of direct and indirect reacting bilirubin present in serum is described. This procedure is a combination of two previously described methods which have been somewhat modified. A comparison has been made between values obtained with the modified procedure and those obtained with such standard methods as the Thannhauser-Andersen modification of the van den Bergh method and the Evelyn and Malloy procedure. Evidence is presented which demonstrates the difference in solubility of the direct and indirect reacting types of bilirubin and it is shown that this difference allows their separate determination. The ratios in which the two types occur are also given. The clinical importance of these two types of bilirubin is to be presented in another paper.

REFERENCES

1. Varela Fuentes, B., and Recarte, P.: Nouvelle technique simplifiée pour le dosage séparé des deux bilirubines, directe et indirecte, des sérums ictériques, Compt. rend. Soc. de biol. 116: 1193, 1934.
2. Varela Fuentes, B., Viana, C., and Recarte, P.: La bilirubine indirecte du sérum et son extraction par le chloroforme. Discussion des techniques proposées par de Castro et par Kerpola et Leikola, Compt. rend. Soc. de biol. 117: 903, 1934.
3. Osterberg, A. E.: The New van den Bergh Reaction for the Determination of Serum Bilirubin Utilizing the Photelometer, J. Lab. & Clin. Med. 22: 729, 1937.
4. van den Bergh, H. A., and Grotepass, W.: An Improved Method for the Determination of Bilirubin in Blood, Brit. M. J. 1: 1157, 1934.
5. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With Photoelectric Colorimeter, J. Biol. Chem. 119: 481, 1937.

6. Griffiths, W. J., and Kaye, Geoffrey: A Study of the Blood-pigment in Obstructive Jaundice, With Observations on the van den Bergh Reaction, Brit. J. Exper. Path. 11: 441, 1930.
7. Grunenberg, Karl: Discussion, Verhandl. d. deutsch. Gesellsch. f. inn. Med. 34: 112, 1922.
8. Andrewes, C. H.: The Nature of the Differences Between the Bilirubins of Obstructive and Haemolytic Jaundice, Brit. J. Exper. Path. 5: 213, 1924.
9. Collinson, G. A., and Fowweather, F. S.: An Explanation of the Two Forms of Bilirubin Demonstrated by the van den Bergh Reaction, Brit. M. J. 1: 1081, 1926.
10. Roberts, W. M.: Observations on the Nature of the van den Bergh Reaction, Brit. J. Exper. Path. 9: 107, 1928.
11. Newman, C. E.: Bilirubin and the van den Bergh Reaction, Brit. J. Exper. Path. 9: 112, 1928.
12. Hunter, George: On the Two Types of Bilirubin Diazo-Reaction in Serum, With a Hypothesis on the Nature of the Bilirubin in the Serum From Haemolytic Jaundice, Brit. J. Exper. Path. 11: 415, 1930.
13. de Castro, Ugo: Neue Methode zur Einzelbestimmung des direkten und indirekten Blutbilirubins, Ztschr. f. d. ges. exper. Med. 67: 673, 1929.
14. Cantarow, A., Wirts, C. W., Jr., and Hollander, G.: Quantitative Studies of Direct-Reacting Serum Bilirubin, Arch. Int. Med. 69: 986, 1942.

STUDIES OF PANCREATIC FUNCTION*

I. The Determination of the Lipolytic Enzymes of Blood Serum

Norman P. Goldstein, M.A., and Joseph H. Roe, Ph.D.
Washington, D. C.

THE DIAGNOSIS of pancreatic disease, other than diabetes mellitus, is difficult, a situation due in part to the limitations of laboratory methods. The laboratory approach to this problem has been along two general pathways: (1) the determination of enzymes of pancreatic origin (amylase, lipase) in the blood serum; (2) the estimation of the amount, bicarbonate content, and enzymes of the external secretion of the pancreas following pancreatic stimulation.

In acute inflammatory lesions of the pancreas, where either a disintegration of pancreatic tissue or obstruction in the ducts causes the back-passage of abnormal quantities of pancreatic enzymes into the blood stream, the determination of the amylase or lipase content of the blood serum has been found very useful. In those lesions of the pancreas where there is a decrease in the amount of enzymes secreted, however, the information obtainable from blood studies is without significance. The indications of an elevation of pancreatic enzymes in the blood serum, in the presence of the other clinical findings, are clear, but the observation in the serum of a lower than normal content of enzymes, which have characteristics similar to those secreted by the pancreas, is difficult to interpret because these enzymes have an extrapancreatic, as well as pancreatic, origin.

---

*From the Department of Biochemistry, School of Medicine, The George Washington University.
Received for publication, May 14, 1943.

In those pancreatic diseases which are characterized by a deficient external secretion, the clinician obtains some information by observations of the stools. Bulky stools, containing undigested muscle fibers, starch, and increased fat, suggest a deficiency in pancreatic secretion. Pratt[1] has stressed the importance of a gross inspection of the stools, and he calls attention to the rather specific relation of creatorrhea to pancreatic insufficiency.

Lagerlöff[2] states that the determination of the enzyme content of the stools is worthless. According to this author, lipase is inactivated in the feces. Moreover, amylase originates in the saliva as well as in the pancreatic juice.

Recently, important advances have been made in the development of laboratory procedures for the direct testing of pancreatic function. Ågren and Lagerlöff[3] appear to be the first investigators to have developed a successful technique for collecting pancreatic juice uncontaminated with saliva or gastric juice. In 1936 these authors described a procedure in which a double lumen tube is introduced into the upper alimentary canal and the contents of the stomach and duodenum are removed simultaneously by suction, before and after the intravenous administration of secretin. Ågren and Lagerlöff, also Diamond and Siegel[4] in this country, have reported that the secretin test gives much valuable information concerning disturbed function of the pancreas, particularly in chronic states.

A new method for evaluating pancreatic function has recently been reported by Free, Beams, and Myers.[5] In this procedure a Rehfuss or Einhorn duodenal tube is introduced into the stomach and the gastric contents are removed. The tube is then passed into the duodenum and a twenty-minute fasting sample of duodenal contents is collected. Twenty-five c.c. of olive oil are then injected slowly through the tube, and after ten minutes siphonage is started again. Three twenty-minute samples of duodenal juice are collected. All samples of duodenal juice are examined for total volume, proteinase, amylase, lipase, and icteric index. Data were obtained upon patients with pancreatic disease which showed marked departures from normal values. These authors report results as good by the use of a single duodenal tube as with a double lumen tube. Exclusion of gastric juice results from the inhibitory effect of olive oil on gastric secretion and motility. This test appears to be on a sound basis and has much in its favor in the way of simplicity and practicality of procedure.

Numerous methods for the determination of lipidolytic enzymes have been proposed. These have involved the use of titration, stalagmometric, manometric, and electrometric techniques. Of these, representative ones will be discussed here. In early reports in the literature, the fatty acid liberated by the hydrolysis of substrate was titrated with standard alkali. In 1911 Rona and Michaelis[6] developed a stalagmometric method, using tributyrin as a substrate. In this method the increase in the number of drops of a solution containing enzyme, substrate, and buffer, due to changes in surface tension, is taken as a measure of enzyme activity. Rona and Lasnitzki,[7] in 1924, devised a manometric method for the estimation of lipase. This method measures manometrically the volume of carbon dioxide released from sodium bicarbonate by the action of butyric acid, the amount of butyric acid being dependent upon the concentration of the

lipase, which promoted the hydrolysis of tributyrin. This method has certain advantages: the pH of the solution is readily maintained and one of the end products of the reaction is continuously removed. A titration method for the determination of lipase was published by Willstätter, Waldschmidt-Leitz, and Memmen[8] in 1923. In this procedure the fatty acid liberated by action of the lipase upon a buffered olive oil emulsion is titrated with standard alkali. In 1932 Cherry and Crandall[9] published a modification of the Loevenhart[10] (1907) method for the determination of esterase, in which the fatty acids resulting from the hydrolysis of a buffered ester or fat emulsion by the enzymes of a biologic material are titrated with twentieth-normal sodium hydroxide. These investigators reported that after ligation of the pancreatic ducts in dogs, they could demonstrate the presence in serum of an olive oil-splitting enzyme (lipase) which normally did not occur in the blood serum. They also found that the activity of an enzyme normally present in blood, as measured by the hydrolysis of ethyl butyrate or tributyrin (esterase), did not show uniform increases following pancreatic duct ligation. They, therefore, concluded that their test for lipase was specific for pancreatic injury. Recently, Troescher and Norris[11] have proposed a method in which the action of the enzyme upon ethyl butyrate in a phosphate buffer solution is measured by the change in pH of the solution due to the butyric acid liberated.

Our interest in this problem arose from a realization of the limitations of present methods for determining the lipidolytic enzymes of the blood. The serum lipase determination has been reported to be especially useful in the diagnosis of pancreatic lesions of the obstructive type.[12-14] The methods used in this work require a 24-hour incubation of serum with substrate. Obviously a procedure for estimating the concentration of an enzyme which requires twenty-four hours of hydrolysis is undesirable from both a theoretical and practical point of view. It is well known that to make an accurate determination of the concentration of an enzyme, one should measure the initial rate of hydrolysis. Furthermore, when a serum lipase determination is desired, the patient usually is acutely ill and waiting for twenty-four hours on an analysis is very objectionable.

Of the various methods proposed for estimating lipase or esterase action, we believe the most suitable procedure for clinical work is the simple titration of the fatty acid liberated by enzymic activity upon a suitable substrate. In attempting to develop a method which would be more accurate and sensitive our attention was directed to a consideration of the following conditions: (1) the substrate used; (2) the emulsifying agent; (3) the pH at which the test is run and therefore the buffers used; (4) removal of the end products of the reaction; (5) the temperature at which the test is carried out; (6) the method of stopping the enzymatic hydrolysis. Our results and conclusions with respect to each of these conditions are set forth below.

SELECTION OF SUBSTRATE

Cherry and Crandall[9] studied the activity of pancreatic and liver extracts and blood serum upon 21 different esters, with particular emphasis upon ethyl butyrate, tributyrin, and olive oil. These investigators abandoned the use of

ethyl butyrate and tributyrin on the grounds that the latter substances were split by blood esterase, whereas only blood lipase splits olive oil. Other investigators, however, have used ethyl butyrate and tributyrin on the same basis as olive oil. Two other new and possible substrates, benzyl butyrate and benzyl stearate, were suggested by the work of Balls and Matlack,[15] who found that the esters of benzyl alcohol are split more rapidly than those of other alcohols. Benzyl stearate was eliminated from use because at ordinary temperatures it is a solid and is extremely difficult to emulsify. The five substrates selected for study were ethyl butyrate, benzyl butyrate, tributyrin, olive oil, and triolein. All five of these exist in the liquid form at room temperatures and are fairly easy to emulsify; two are simple esters and three are glycerides, a selection which appeared to offer an opportunity to study the question of whether the biologic fluid being examined contains an esterase, a lipase, or both. Triolein was selected in order to have a pure triglyceride of a known long chain fatty acid for comparison with olive oil, an impure mixture of glycerides and other substances.

TABLE I

RATE OF SPLITTING OF DIFFERENT SUBSTRATES

SUBSTRATE	GLYCEROL EXTRACT OF PANCREAS	GLYCEROL EXTRACT OF LIVER	HUMAN SERUM	CAT SERUM	RABBIT SERUM	DOG SERUM
Ethyl Butyrate (0.25 c.c.)	5.20 (1 hr.)	4.67 (1 hr.)	0.33 (24 hr.)	0.41 (24 hr.)	0.28 (24 hr.)	1.90 (24 hr.)
Tributyrin (0.25 c.c.)	13.40 (1 hr.)	2.68 (1 hr.)	0.83 (1 hr.)	1.01 (1 hr.)	1.19 (3 hr.)	1.95 (1 hr.)
Benzyl Butyrate (0.25 c.c.)	9.77 (1 hr.)	6.52 (1 hr.)	0.49 (3 hr.)	0.65 (3 hr.)	0.53 (3 hr.)	2.19 (3 hr.)
Olive Oil (1.00 c.c.)	6.24 (1 hr.)	00 (24 hr.)	0.18 (24 hr.)	0.26 (24 hr.)	0.22 (24 hr.)	0.38 (24 hr.)
Triolein (0.25 c.c.)			0.15 (24 hr.)	0.31 (24 hr.)		0.33 (24 hr.)

Expressed as c.c. of 0.1 N alcoholic KOH per c.c. of serum or c.c. of tissue-glycerol preparation. Parentheses show amount of substrate in column 1 and time of hydrolysis elsewhere.

The results of this study are recorded in Table I. Our data show that the greatest amount of hydrolysis is obtained with tributyrin as a substrate in experiments with glycerol extract of pancreas and with human, cat, and rabbit serum; whereas with glycerol extract of liver, benzyl butyrate gave a higher value. These results suggest that tributyrin should be the substrate of choice to obtain the most sensitive test when analyzing for enzymes of pancreatic origin and that benzyl butyrate would be the most sensitive substrate for enzymes of liver origin.

THE EMULSIFYING AGENT

For emulsifying agents a number of substances have been used for measurement of lipidase action. Cherry and Crandall[6] favored acacia; Willstätter, Waldschmidt-Leitz, and Memmen[4] used albumin; and Balls, Matlack, and Tucker[16] have utilized a glycerol solution of ox bile. We carried out a series of estimations to determine the most effective emulsifying agent. The data of

Table II show that the bile-glycerol mixture, prepared according to Balls, Matlack, and Tucker,[16] is the most favorable emulsifying agent when benzyl butyrate is used as substrate; in view of these results it was decided to use bile as the emulsifying agent in our experiments.

THE pH AND SELECTION OF BUFFERS

Balls, Matlack, and Tucker[16] observed that pancreatic lipase does not appear to have a very sharp pH optimum. Experiments with tristearin as substrate and NH_3—NH_4Cl mixture as buffer at pH 8.2 to 8.4 and 7.2 to 7.5 showed very little difference in the rates of hydrolysis. Willstätter and Waldschmidt-Leitz[17] found the optimum pH values for pancreatic lipase were 9.2 and 8.3 with olive oil and tributyrin, respectively, as the substrates. With tripropionin as substrate, Weinstein and Wynne[18] found the optimum pH is 7.2 with phosphate-borate buffer and 9.3 with a glycine buffer. Anrep, Lush, and Palmer,[19] using a phosphate buffer and glycerol triacetate as substrate, began their hydrolysis at a pH of 7.8 and permitted a shift in pH to 7.0. They considered a pH below 7.0 to be too low for satisfactory quantitative studies with lipase.

TABLE II

EMULSIFYING POWER OF DIFFERENT EMULSIFYING AGENTS

SUBSTRATE	BILE (DIFCO)	NA. TAURO-CHOLATE (MERCK)	NA. TAURO-CHOLATE (PFANSTIEHL)	NA. GLYCO-CHOLATE (PFANSTIEHL)	FRESH EGG ALBUMIN SOLUTION	DRIED EGG ALBUMIN SOLUTION	5 PER CENT DUPONOL SOLUTION	5 PER CENT ACACIA SOLUTION
Benzyl Butyrate (0.240 Gm.)	11.01	5.75	8.56	5.60	1.99	2.55	0.43	3.21

Expressed as c.c. of 0.1 N alcoholic KOH using 5 c.c. of the emulsifying agent and 1 c.c. of glycerol preparation of calf pancreas. Time of hydrolysis was one hour.

From the work discussed above, it appears that one should carry out the hydrolysis at a pH somewhere within the range of 7.0 to 9.2. In view of the observations mentioned, we decided to fix the buffering conditions so that the initial pH is 8.2 to 8.4, and the pH at the conclusion of the hydrolysis is above 7.0. Under the conditions of our procedure, some shifting in pH is permitted, but this is desirable to avoid using excess quantities of buffer which would have an inhibiting effect. The buffer we found most suitable for our work was sodium diethyl barbiturate, and the amount we determined to be most effective was 0.05 Gm. in the final volume of the test used which is 26 c.c.

In the hydrolysis mixture set up for our method 3 c.c. of 0.1 N butyric or oleic acid are required to shift the pH from 8.4 to 7.0. The buffering system in this procedure, therefore, has the capacity to permit optimum buffering with blood sera having concentrations of lipidolytic enzymes up to 300 units per 100 c.c. If concentrations greater than the latter are encountered, the determination may be repeated, using less serum, to obtain greater accuracy.

In Table III are presented data which show the effectiveness of the buffering system we use in holding the pH within a satisfactory range. This is an important feature of our method; the pH at the end of the hydrolysis is still

in the favorable range, unless very high concentrations of enzymes are encountered, in which case a smaller sample of serum is analyzed. In the Cherry and Crandall method, which has received widespread clinical use in this country, a phosphate buffer mixture with a pH of 7.0 is employed. This is at the lower margin of the pH range recommended by other authors for pancreatic lipase; furthermore, the fatty acid liberated during the hydrolysis shifts the pH to a still lower value, resulting in a corresponding decrease in the sensitivity of this test.

TABLE III

CHANGES OF PH DURING HYDROLYSIS

SUBSTRATE	SERUM	TIME OF HYDROLYSIS	CONTROL PH	PH AFTER HYDROLYSIS
Ethyl Butyrate (0.25 c.c.)	Cat	24 hr.	8.20	7.64
Ethyl Butyrate (0.25 c.c.)	Human	24 hr.	8.20	7.95
Tributyrin (0.25 c.c.)	Cat	3 hr.	8.16	6.20
Tributyrin (0.25 c.c.)	Human	1 hr.	8.31	7.90
Benzyl Butyrate (0.25 c.c.)	Cat	3 hr.	8.15	7.55
Benzyl Butyrate (0.25 c.c.)	Human	3 hr.	8.30	8.06
Olive Oil (1.00 c.c.)	Cat	24 hr.	8.15	7.63
Olive Oil (1.00 c.c.)	Human	24 hr.	8.22	8.09

Using 50 mg. of sodium diethylbarbiturate for each substrate in a hydrolysis with 1 c.c. of serum. Measurements made with a glass electrode.

REMOVAL OF THE END PRODUCT OF HYDROLYSIS

Willstätter, Waldschmidt-Leitz, and Memmen,[8] in 1923, instituted the use of calcium chloride to aid the hydrolysis of a fat by lipase. They attributed their favorable results to the formation of an adsorption compound, fat-calcium-albumin-lipase. Balls, Matlack, and Tucker[16] have continued the use of calcium chloride to aid the reaction. These investigators offer the much more reasonable explanation that the calcium ion combines with the fatty acid liberated in the hydrolysis to form an unionized or insoluble calcium salt. This removal of one of the end products prevents the reversal of the reaction and thus promotes a rapid splitting of the substrate. We adopted the use of the calcium ion, but in our work it was found that the chloride ion of calcium chloride, which was released with the formation of the calcium salt of the fatty acid, markedly reduced the pH of the medium, slowing down the reaction. To avoid this untoward effect, several calcium salts of weaker acids were tested. Calcium acetate was selected as the preferred salt to obtain the calcium ion effect of removal of one of the end products of the reaction and, at the same time, to promote good buffering. Balls, Matlack, and Tucker[16] reported that too much calcium chloride is somewhat inhibitory to this reaction. This effect was not related to lowering of the pH, as these authors maintained a fairly constant pH by the addition of ammonia. We found 0.2 Gm. of calcium acetate in 26 c.c. of solution to be the optimum amount for this reaction. This quantity provides for the fatty acid liberated by blood serum extraordinarily rich in lipase and at the same time does not have an inhibitory effect.

INACTIVATING THE ENZYME

Among the various methods of inactivating an enzyme, the two that are most convenient for an analytic method of this type are inactivation by heat and by alcohol. An important point, however, is that the control should be inactivated by the same method as the experimental. In some methods there exists the error of inactivating the control tubes containing the serum by heating and the experimental tubes containing the hydrolysate by addition of alcohol. The error that may occur from inactivating the same serum by the two methods is illustrated by Table IV.

TABLE IV

DIFFERENCE IN TITRATION VALUE OF SERUM ACCORDING TO METHOD OF INACTIVATION

SERUM	SUBSTRATE	METHOD OF INACTIVATION	C.C. OF 0.1 N KOH
Cat 3	----	Heating in Boiling H_2O	1.78
Cat 3	----	Addition of 100 c.c. of Alcohol-ether Mixture	2.25
Cat 2	Benzyl Butyrate	Heating in Boiling H_2O	1.48
Cat 2	Benzyl Butyrate	Addition of 100 c.c. of Alcohol-ether Mixture	2.47

The results of Table IV show the fallacy of inactivating the control tube by one method and the experimental by another method. The discrepancy exhibited in these data will account for a part of the values found in blood sera by the methods which use the two different procedures for inactivation.

Following the work of Balls, Matlack, and Tucker,[16] we have used a mixture of 9 volumes of 95 per cent alcohol and 1 volume of ether to inactivate both our experimental and control tubes. The alcohol, in addition to inactivating the lipase, provides an excellent medium for titration of the fatty acid released by the enzymatic hydrolysis, suppressing the dissociation of the hydroxyl ions in the mixture, and thus permitting the titration of the fatty acid.

TEMPERATURE AND INDICATOR

Balls, Matlack, and Tucker[16] have found that the optimum temperature for lipase action is around 40 to 50° C. We selected the temperature of 37° C. for our procedure because most laboratories usually keep a water bath at that temperature.

The indicator chosen for this work was phenolphthalein, since this indicator is suitable for titration of a weak acid with a strong base. It is of interest that the end point of this titration, as measured by the glass electrode, is at a pH around 10.2, which is higher on the pH scale than in an aqueous titration with phenolphthalein.

PROPOSED METHOD FOR LIPASE OR ESTERASE DETERMINATION

Reagents

1. *Glycerol-bile emulsifying reagent.*—(Balls, Matlack, and Tucker.) To 50 Gm. of dried oxbile (Bacto-oxgall, Difco Laboratories) in a liter Erlenmeyer flask add 50 c.c. of water. Heat this solution in an autoclave at 15 pounds pressure for two hours. Cool, add 500 c.c. of glycerol, and heat the mixture on a

steam bath until a clear solution is obtained. Keep in a stoppered flask in the ice box. When used, the flask is warmed in a water bath to 60 to 70° C. to facilitate pipetting.

2. *Buffer.*—Dissolve 5 Gm. of sodium diethylbarbiturate in 1 liter of solution.

3. *Calcium acetate solution.*—Dissolve 20 Gm. of calcium acetate, chemically pure $Ca(CH_3COO)_2 \cdot H_2O$, in 1 liter of solution.

4. *Alcohol-ether inactivating mixture.*—To 900 c.c. of 95 per cent ethyl alcohol add 100 c.c. of ether.

5. *Standard alkali.*—A 0.1 N alcoholic solution of potassium hydroxide is used.

Procedure

The Experimental.—Pipette the required amount of the substrate (0.25 c.c. of ethyl n-butyrate, tributyrin, benzyl n-butyrate, olive oil, or triolein) into a large eight-inch test tube. Into this tube are pipetted 5 c.c. of the bile-glycerol solution, previously heated in a water bath to 60 to 70° C. for convenience in pipetting. The tube is shaken, and then placed in boiling water for five to ten minutes, after which it is shaken again until the substrate is completely emulsified. After cooling to room temperature, pipette 10 c.c. of calcium acetate (200 mg.) and 10 c.c. of sodium diethylbarbiturate (50 mg.) into the tube. The tube is then placed in a water bath at 37° C., and when the mixture has reached the latter temperature, 1 c.c. of blood serum is pipetted into the tube and mixed thoroughly with the contents by means of a stirring rod. After the appropriate lapse of time (one hour for tributyrin, three hours for benzyl butyrate, twenty-four hours for olive oil, triolein, and ethyl butyrate), pour the contents of the tube into a 250 c.c. Erlenmeyer flask containing 100 c.c. of alcohol-ether mixture. Rinse the empty test tube with the alcohol-ether digestion mixture and pour the rinsings back into the Erlenmeyer flask. Add approximately 1 c.c. of 1 per cent phenolphthalein solution, and titrate with 0.1 N alcoholic potassium hydroxide to the same shade of pink as the blank. It is best to titrate to a deep permanent pink.

The Blank.—The blank tube is prepared and treated in exactly the same way, except that addition of the substrate is omitted. The substrate used should be neutral, and hence a correction for the omission of substrate in the blank is not needed.

Calculation.—Titration of experimental − titration of blank × 100 = units of lipase or esterase per 100 c.c. of serum.

We have redefined the lipase or esterase unit, and in agreement with conventional usage, have expressed results in terms of amount per 100 c.c. of serum. A unit of lipase or esterase is the amount of enzyme that will liberate 1 c.c. of 0.1 N fatty acid from an appropriate substrate at 37° C. in the time allowed (tributyrin, one hour; benzyl butyrate, three hours; ethyl butyrate, olive oil, triolein, twenty-four hours).

DISCUSSION

Using the improved conditions for enzyme activity which have been introduced into the above procedure, we obtain as high a titration in one hour's

hydrolysis of tributyrin by blood serum as is secured by twenty-four hours' hydrolysis of olive oil with the same serum. If tributyrin hydrolysis is as good a measure of the pathology present in pancreatic disease as olive oil hydrolysis, it would be a marked advance to use this one-hour test with tributyrin as substrate. To study this possibility, experiments with animals have been carried out and will be reported in another paper.[20] We may say here that in those experiments which were designed to simulate pancreatic pathology (pancreatic duct ligation, mecholyl stimulation, pancreatectomy), results obtained by the one-hour test with tributyrin as the substrate have reflected the condition present just as characteristically as when a 24-hour hydrolysis of olive oil was carried out. Chloroform poisoning, however, gave a dissociation of results: that is, an increase in the tributyrinase content of the serum without an increase in the values obtained with olive oil or triolein as substrates. Such results raise a question as to whether or not tributyrinase activity of serum might arise from liver injury as well as from pancreatic disease. The only way this question can be answered is by clinical trials. Our clinical studies thus far are too few to justify any conclusions at this time.

Following the above method with tributyrin as the substrate, we determined the lipase in the blood serum of a group of 30 apparently normal medical students. The values obtained ranged from 35 to 112 units per 100 c.c. of serum, showing a normal distribution curve with a peak at about 65 units. Nine of these values were obtained upon bloods collected in the morning about fifteen hours after food intake, and the remaining values are for bloods collected in the afternoon from individuals in the postabsorptive state about six hours. Some variation during the day appears to occur in the lipase content of blood serum, which, however, is not very marked. In our hands duplicate determinations with this method agreed within ±4.5 tributyrinase units per 100 c.c. of serum.

In all of our studies, a more rapid splitting of substrate was obtained with tributyrin than with the other four substrates used, namely, ethyl butyrate, benzyl butyrate, triolein, and olive oil. In explanation of this, it is suggested that the response measured with tributyrin represents the action of more than one enzyme; possibly tributyrin is attacked by "esterase" because of its short chain fatty acids, and at the same time is split by "lipase" since it is a triglyceride.

Since olive oil is a mixture of different glycerides and other substances and its composition may be expected to vary according to its source and methods of preparation, the use of this substrate in estimating "lipase" activity is to be questioned. For this reason we made parallel studies with a highly purified triolein* preparation as a substrate. The results obtained with triolein and olive oil as substrates, however, compare fairly closely, quantitatively, and have a similar significance in the studies made.

With the procedure outlined above, we have consistently found an enzyme in the blood of the normal dog, cat, rabbit, and human being which splits the lipids of olive oil. This observation is not in agreement with the work of Cherry and Crandall,[9] who did not find an enzyme in the blood of the normal dog which

*Kindly supplied to us by Dr. G. S. Jamieson of the Bureau of Agricultural Chemistry and Engineering, U. S. Dept. of Agriculture.

splits olive oil. We believe the inability of the Cherry and Crandall test to demonstrate the presence of an olive oil-splitting enzyme in normal dog serum is due to the lack of sensitivity of this method. In the latter procedure the hydrolysate is buffered to produce an initial pH of 7, which is below the optimum range for lipase; this feature would reduce considerably the rate of hydrolysis and could account for the failure to obtain splitting of olive oil by normal dog serum.

After our comparative studies were under way, it was realized that tributyrin could be dispersed without the aid of bile, since this glyceride is slightly soluble in water. Experiments showed that tribuytrin, when properly homogenized in the aqueous buffered digestion mixture, is split by blood serum even more rapidly than when emulsified with bile. It was necessary to use bile in our comparative studies for the satisfactory emulsification of the other substrates, and to make a true comparison, tributyrin had to be emulsified in the same way. Certain advantages are to be gained by using an aqueous tributyrin suspension, however. The end point of the titration with the aqueous suspension is more easily discerned, and the difficulty of preparing and titrating a bile emulsion is obviated. If bile is not used as the emulsifying agent for tributyrin, an equal volume of water is substituted and the test is carried out in the same way. This change in technique will yield values for normal blood serum about twice as high as those we obtained with the use of bile as the dispersing agent. We plan to report later normal values for human serum by this method when an aqueous tributyrin suspension is used.

The relation of the amount of fatty acid liberated to the concentration of enzyme and to the time of hydrolysis in the proposed method is of interest. As twenty-four hours of hydrolysis are required when ethyl butyrate or triolein or olive oil is used as the substrate, and three hours of hydrolysis are required with benzyl butyrate as substrate, studies of time or concentration relationships would be somewhat unsatisfactory with these substrates. The tributyrin procedure, however, is well adapted to such studies, since it requires only one hour of hydrolysis. The studies we made were with tributyrin as substrate, the procedure being the same as outlined above.

The results of these studies are shown in Figs. 1 and 2. Exact linear relationship of the amount of fatty acid liberated to the time of hydrolysis, or to the concentration of enzyme, is not shown. Fairly good relationships are present in the first three hours of hydrolysis, however; also where small amounts of enzyme (0.5 c.c. and 1 c.c. of serum) are used.

The amount of substrate used in these studies is a marked excess. The 0.25 c.c. of tributyrin is equivalent to 26 c.c. of 0.1 N butyric acid; the same amount of triolein is equivalent to 7.4 c.c. of 0.1 N oleic acid. These amounts of fatty acid are 25 to 30 times that liberated in a hydrolysis by the enzymes of normal human serum under the conditions of the method described. The pH at the end of the hydrolysis in the experiment represented by Fig. 1 was 7.3; in the experiment of Fig. 2 the pH at the end of three hours was 7.4. Both of these pH values are within the optimum range. The deviations from straight line relationships in Figs. 1 and 2 must therefore be attributed to other conditions than the amount of substrate or the buffering of the reaction.

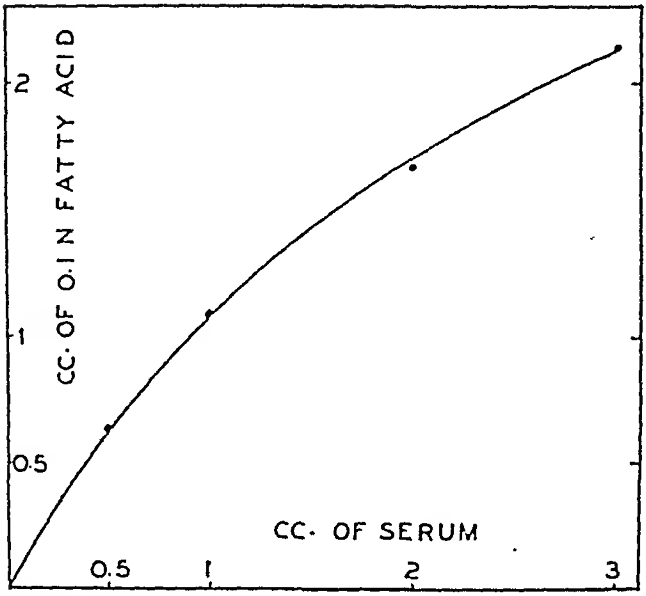


Fig. 1.—Relation of amount of fatty acid liberated to the concentration of enzyme under the conditions of the proposed method. Tributyrin was used as substrate, the time of hydrolysis being one hour. Human serum was the source of enzyme.

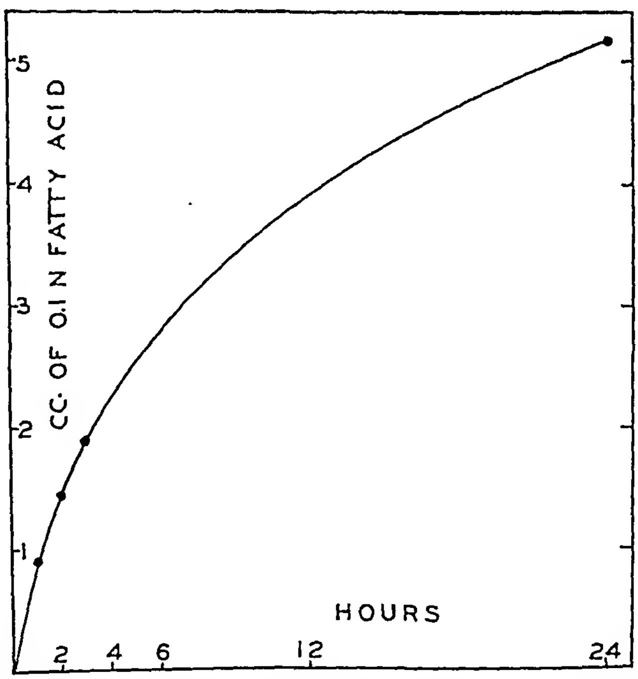


Fig. 2.—Relation of amount of fatty acid liberated to the time of hydrolysis under the conditions of the proposed method. Tributyrin was used as substrate, and cat serum was the source of enzyme.

SUMMARY

1. A study of the conditions entering into the determination of the lipidolytic enzymes of blood serum has been made. Attention was directed to the substrate, the emulsifying agent, the pH and suitable buffering, and the removal of end products.

2. In comparative studies of the five substrates, ethyl butyrate, tributyrin, benzyl butyrate, triolein, and olive oil, it was found that tributyrin is split most rapidly by the enzymes of blood serum.

3. A method for the estimation of the lipidolytic enzymes of blood serum is proposed. Using tributyrin as the substrate in this method, the amount of fatty acid liberated in one hour's hydrolysis by blood serum is comparable to the quantity obtained when a 24-hour hydrolysis of olive oil by the same serum is carried out.

4. This rapid test, involving a one-hour hydrolysis of tributyrin, may have clinical advantages. Further clinical work is necessary to appraise its possibilities.

REFERENCES

1. Pratt, J. H.: Pancreatic Disease: The Frank Billings Lecture, J. A. M. A. 120: 175, 1942.
2. Lagerlöff, H.: Pancreatic Function and Pancreatic Disease, New York, 1942, The Macmillan Company, p. 123.
3. Agren, G., and Lagerlöff, H.: Pancreatic Secretion in Man After Intravenous Administration, Acta. med. Scandinav. 90: 1, 1936.
4. Diamond, J. S., and Siegel, S. A.: The Secretin Test in the Diagnosis of Pancreatic Diseases With a Report of One Hundred-Thirty Tests, Am. J. Digest. Dis. 7: 435, 1940.
5. Free, A. H., Beams, A. J., and Myers, V. C.: Studies of the Enzyme Activities of Duodenal Contents as a Means of Evaluating Pancreatic Function, Gastroenterologia 1: 188, 1943.
6. Rona, P., and Michaelis, L.: Upon Ester and Fat-Splitting in Blood and Serum, Biochem. Ztschr. 31: 345, 1911.
7. Rona, P., and Lasnitzki, A.: A Method for the Determination of Lipase in Body Fluids and Tissues, Biochem. Ztschr. 152: 504, 1924.
8. Willstätter, R., Waldschmidt-Leitz, E., and Memmen, F.: Pancreatic Enzymes. I. Determination of Pancreatic Fat Hydrolysis, Ztschr. f. physiol. Chem. 126: 93, 1923.
9. Cherry, I. S., and Crandall, L. A., Jr.: The Specificity of Pancreatic Lipase: Its Appearance in the Blood After Pancreatic Injury, Am. J. Physiol. 100: 266, 1932.
10. Loevenhart, A. S.: On the So-Called Coferments of Lipase, J. Biol. Chem. 2: 391, 1907.
11. Troescher, E. E., and Norris, E. R.: A Micro Blood Esterase Determination Applied to Studies of Rats Bearing Adenocarcinoma, J. Biol. Chem. 132: 553, 1940.
12. Comfort, M. W., and Osterberg, A. E.: Lipase and Esterase in the Blood Serum: Their Diagnostic Value in Pancreatic Disease, J. Lab. & Clin. Med. 20: 271, 1934.
13. Comfort, M. W.: Serum Lipase: Its Diagnostic Value, Am. J. Digest. Dis. 3: 817, 1937.
14. Comfort, M. W., and Osterberg, A. E.: The Value of Determination of the Concentration of Serum Lipase and Serum Amylase in the Diagnosis of Disease of the Pancreas, Proc. Staff Meet., Mayo Clinic 15: 427, 1940.
15. Balls, A. K., and Matlack, M. B.: The Enzymic Hydrolysis of Benzyl Stearate and Benzyl Butyrate, J. Biol. Chem. 125: 539, 1938.
16. Balls, A. K., Matlack, M. B., and Tucker, I. W.: The Hydrolysis of Glycerides by Crude Pancreas Lipase, J. Biol. Chem. 122: 125, 1937.
17. Willstätter, R., and Waldschmidt-Leitz, E.: Pancreatic Enzymes. II. Pancreatic Lipase, Ztschr. f. physiol. Chem. 126: 132, 1923.
18. Weinstein, S. S., and Wynne, A. M.: Studies on Pancreatic Lipase. I., J. Biol. Chem. 112: 641, 1935.
19. Anrep, G., Lush, J. L., and Palmer, M. G.: Observations on Pancreatic Secretion, J. Physiol. 59: 434, 1925.
20. Goldstein, N. P., and Roe, J. H.: Studies of Pancreatic Function. II. The Effect of Injury to the Pancreas or Liver Upon the Amylase and Lipidase Content of the Blood, J. Lab. & Clin. Med. 28: 1334, 1943.

A SIMPLE METHOD FOR THE APPROXIMATE ANALYSIS OF CARBON DIOXIDE AND OXYGEN IN GAS SAMPLES*

ALBERTA ILIFF, PH.D., GLADYS M. KINSMAN, PH.D.† ROBERT M. HILL, PH.D., AND ROBERT C. LEWIS, PH.D.
DENVER, COLO.

A SIMPLE apparatus which can be used by a relatively inexperienced technician to determine the carbon dioxide and oxygen content of samples of gas has been developed. The method of analysis is rapid and suitable for determinations where an accuracy of 0.3 per cent is adequate. This apparatus may be used for the determination of oxygen in samples of gas taken from cylinders containing mixtures of oxygen and nitrogen, and for the determination of carbon dioxide and oxygen in samples of gas collected from anesthetic apparatus, from oxygen tents, or from the thoracic cavity of patients with pneumothorax.

APPARATUS

The apparatus (Fig. 1) consists of a 10 c.c. burette calibrated in 0.05 c.c., on the top of which a stopcock (*A*) and a cup (*B*) have been annealed. It is advisable to have on hand at least two of these burettes in order that duplicate determinations can be made simultaneously.

The other pieces of equipment necessary to make the determinations consist of three 2,000 c.c. graduated cylinders, or hydrometer cylinders of similar size, in which the burettes may be completely immersed; three medicine droppers with fine capillary tips for filling the cups of the burettes and the rubber tubing connections; a ring stand equipped with a double spring burette clamp to support the burettes while introducing the solutions; a mouthpiece with a six-inch piece of rubber tubing attached to be used in filling the burettes with water; a 50 c.c. syringe and an appropriate needle for the collection of samples; and two short pieces (1.5 and 4 inches) of transparent rubber tubing and a screw clamp for use in the transfer of gas from the syringe to the burette or from a cylinder of oxygen and nitrogen directly to the burette.

CALIBRATION OF APPARATUS

In order to know the total amount of gas in the sample to be analyzed, it is necessary to determine the volume of the uncalibrated portion *C* (Fig. 1) of the burette between the zero mark and the stopcock *A*. This may be done by weighing the amount of water which the uncalibrated portion *C* of the burette will hold. To fill the burette, a short piece of rubber tubing and a mouthpiece, similar to that used with hematological pipettes, is attached to the capillary tip *D* below the stopcock *E*. The cup *B* is immersed in a beaker containing freshly

*From the Department of Biochemistry and the Child Research Council, University of Colorado School of Medicine.

†Formerly of this staff, now Professor of Nutrition, Department of Home Economics, University of Illinois, Urbana.

Received for publication, May 13, 1943.

boiled distilled water, which is drawn up by suction until the burette is completely filled. Care must be taken to prevent the presence of any air bubbles in the burette. Stopcocks *A* and *E* are closed, and the burette is placed in a burette clamp with the cup end up. Stopcock *A* is then opened, and, by careful control of stopcock *E*, just enough water is released so that the bore of stopcock *A* will contain air and the rest of the burette will be completely filled with water. The water is then discharged into a weighing bottle until the level of the liquid reaches the zero mark. The weight of the water and the temperature at which the weighing is made are recorded. An average of ten such weighings is corrected to the density of water at 25° C., and this value is used in the computation of the volume of the portion *C*.

The accuracy of the calibrations on the burette may be checked by the usual method of weighing the amount of water contained in each cubic centimeter.

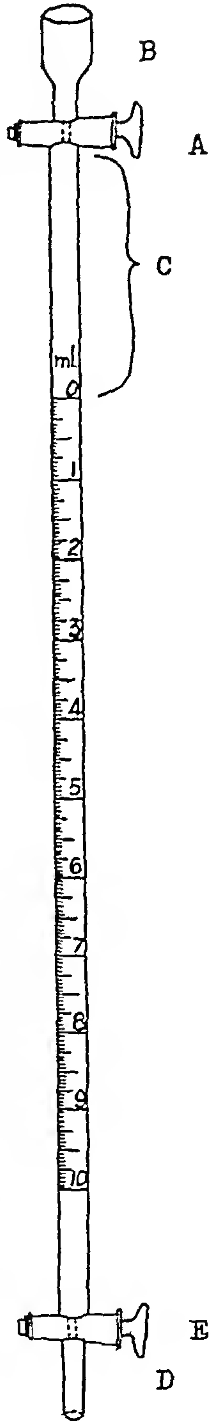


Fig. 1.—Diagram of burette used for the determination of carbon dioxide and oxygen in samples of gas. 1/3 actual size.

SOLUTIONS

A 15 per cent solution of either sodium or potassium hydroxide is used for the absorption of carbon dioxide. The potassium pyrogallate solution,[1] which is used for the absorption of oxygen, is prepared as follows: 450 Gm. of potassium hydroxide are dissolved in 300 c.c. of water, and, after complete solution has occurred, the liquid is thoroughly stirred, heated to boiling, and allowed to stand until it reaches room temperature. Then, 400 c.c. of this potassium hydroxide solution are added to 40 Gm. of pyrogallic acid in a 16 oz. widemouthed dark glass bottle. This is immediately stoppered, sealed with paraffin, and allowed to stand for a week before use. Since the solution keeps for two years or longer under these conditions, several bottles may be prepared at one time. When a bottle is opened for use, a rubber-stoppered washing bottle assembly is substituted for the original stopper. To prevent deterioration of the potassium pyrogallate between analyses, the ends of the glass tubing which project out through the stopper are protected from the air by medicine dropper bulbs. If care is thus taken to prevent undue exposure to air, this solution will keep in the washing bottle for at least nine months.

An oxygen absorbent,[2] which may be used immediately after it is made, consists of 16 Gm. of sodium hyposulfite, 13.3 Gm. of sodium hydroxide, 4 Gm. of anthraquinone β-sulfonic acid and 100 c.c. of water. When freshly prepared, it is just as satisfactory as the potassium pyrogallate. This alternate absorbent deteriorates rather rapidly, however,

even though it is protected from exposure to air by keeping it under mineral oil. Within one or two weeks a heavy precipitate develops during the analysis and thus prevents an accurate reading of the level of the solution.

COLLECTION OF GAS FOR ANALYSIS

The sample for analysis may be collected in a syringe and transferred to the burettes, or it may be taken directly into the burettes. The former procedure is used for the collection of pneumothorax air or of gas from an anesthetic apparatus; the latter, for the collection of air from an oxygen tent or from a tank containing a mixture of nitrogen and oxygen.

When a sample of pneumothorax air for duplicate analysis is to be collected, a sterile needle and a sterile 50 c.c. syringe must be used. The needle and the tip of the syringe are filled by drawing 1 or 2 c.c. of sterile water into the syringe, expelling any air bubbles that may have been admitted, and then emptying the syringe of water. The needle and the tip of the syringe are thus left filled with sterile water. The needle is inserted into the chest wall, and air is drawn into the syringe. After the needle is withdrawn, it is quickly removed from the syringe and a short piece of transparent rubber tubing, which has previously been clamped in the middle and completely filled with water, is immediately slipped onto the tip of the syringe. The transparency of the tubing permits one to be sure that no contaminating air bubbles have been admitted. After filling the burette with water in the manner described under the directions for its calibration, the unattached end of the rubber tubing, which should still be filled with water, is carefully slipped over the capillary tip *D*. Again care must be taken to exclude all air bubbles. The clamp on the rubber tubing is released and both stopcocks of the burette are opened while the cup end is held lower than the syringe. By pressing lightly on the plunger of the syringe, a sample of gas is transferred to the burette. When only about 4 c.c. of water remain in the burette, stopcock *A* and then stopcock *E* are closed. The tubing is clamped off and then removed from the burette. After again filling the free end of the tubing with water, a duplicate sample is transferred from the syringe to a second burette.

When gas from an anesthetic apparatus is to be collected, the needle and syringe need not be sterilized. The needle and the tip of the syringe are filled with water, and a sample of air is secured by inserting the needle in one of the rubber tubing connections. From this point, the procedure for transferring the gas to the burettes is the same as that described for pneumothorax air.

Air from an oxygen tent is collected directly into the burette. After the burette has been completely filled with water, the stopcock *E* is opened, and this end of the burette is inserted through the armhole of the tent to a point about midway between the face of the patient and the front wall of the tent. The burette is held at an angle of about 45 degrees, and the air sample is collected by permitting the water to run out of the lower stopcock *A* until about 4 c.c. remain. Stopcock *E* is closed as the burette is being withdrawn from the tent. In a similar manner, a duplicate sample is collected in a second burette.

In sampling gas from a tank containing a mixture of nitrogen and oxygen, a four-inch piece of transparent rubber tubing is connected to the outlet of the

tank. Room air is washed out of the valve of the tank by gently releasing gas for five or six seconds. The rubber tubing and the burette are completely filled with water, and the free end of the tubing is connected to the burette at tip *D*. If care is taken, this may be done without the introduction of room air. After opening stopcocks *A* and *E*, all but about 4 c.c. of water are forced out of the burette by a gentle stream of gas. The valve of the tank is shut off and the burette is disconnected after both stopcocks have been closed. In the same way a duplicate sample of gas is collected in a second burette.

PROCEDURE FOR ANALYSIS

The burettes containing the duplicate samples of gas are placed in the clamps with the cup ends uppermost. Approximately 2 c.c. of water are introduced into each cup with a medicine dropper. The upper stopcock of each burette is opened, and the lower stopcock *E* is then cautiously turned to permit enough water to run out to fill the capillary tip *D* with water. The lower stopcock must be closed while sufficient water remains in cup *B* to prevent the entrance of room air into the sample. After removing the excess water from the cup with a medicine dropper, the lower stopcock *E* of the burette is immersed in the water which almost fills the first graduated cylinder; the stopcock is opened; and the burette is lowered into the cylinder so that the entire burette up to the cup is covered with water. The two burettes are thus left in the water for two minutes in order that the gas may attain the same temperature as that of the water. In turn, each burette is lifted by the cup to prevent heating of the gas by the warmth of the hand, the exposed part is dried off with cheesecloth, and the height of the burette is adjusted so that the level of the water inside the burette is the same as that in the cylinder. The top (outer border) of the meniscus is read, because the bottom of the meniscus cannot be clearly distinguished later in the analysis after potassium pyrogallate is added. After the burette is read, the lower stopcock *E* is closed under water; the burette is withdrawn and again placed in the burette clamp. The second burette is handled in a similar manner. Approximately 2 c.c. of the potassium hydroxide solution are now admitted into each burette in the same way as the water was introduced. The burettes are removed from the clamps and inverted twice to assure contact of the gas with the potassium hydroxide. The burettes are then placed in the second graduated cylinder, and, after permitting all of the solution in each to drain to the lower end, readings are made as previously described. The burettes are removed, again inverted, returned to the graduated cylinder, and read a second time. If the two readings do not check within 0.02 c.c., the process is repeated. The difference between the initial and the final reading multiplied by 100 and divided by the sum of the initial reading and the volume of the uncalibrated portion *C* of the burette gives the per cent of carbon dioxide in the sample.

When check readings for carbon dioxide have been obtained, about 2 c.c. of potassium pyrogallate solution are blown into the cup of each burette from a bottle of this reagent that has been fitted with a washing bottle assembly. The pyrogallate is admitted to the burettes in the same manner as described for the

other solutions. The burettes are inverted twice, placed in the third graduated cylinder, and read as previously described. The process of inversion and reading is repeated until no change greater than 0.02 c.c. occurs in either burette on successive readings. The per cent of oxygen in the sample is calculated by taking the difference between the final reading for carbon dioxide and the final reading for oxygen, multiplying this difference by 100, and dividing by the sum of the initial reading (before the absorption of any gas has taken place) and the volume of the uncalibrated portion *C* of the burette.

When the analysis is completed, the burettes are thoroughly rinsed until the wash water no longer gives an alkaline reaction to litmus paper. Further analyses may be made immediately. From twenty to twenty-five minutes are required for making duplicate analyses and for washing the burettes in preparation for the next determination. If only one pair of duplicate analyses is to be made, just one cylinder needs to be used, since the loss of solutions into the water will not impair the accuracy of the next step in the determination. When the analyses for the day are completed, the stopcocks of the burettes are carefully greased and the cylinders are washed and filled with water, so that the air in the water will be lost by the time the next determinations are to be made.

ACCURACY OF THE METHOD

Table I gives results of analyses obtained on several different types of determinations and shows the variation to be expected by this method of analysis. It may be seen that the variation observed between duplicate analyses is not greater than 0.35 per cent. As a further check on the method, air from an open circuit chamber procedure for the determination of basal metabolism[3] has been analyzed both with the Carpenter-Haldane gas analysis apparatus[4] and with the procedure herein reported. Table II gives the results of 21 such analyses. The values found for carbon dioxide and for oxygen with the apparatus described check within 0.5 per cent of one another, and the mean values are within ±0.3 per cent of those obtained with the Carpenter-Haldane apparatus. It is evident from the results reported in Tables I and II that the method of analysis is suitable for determinations where an accuracy of 0.3 per cent is adequate.

TABLE I

ANALYSIS OF GAS FROM VARIOUS SOURCES BY THE SIMPLIFIED PROCEDURE DESCRIBED

SAMPLES FROM	CARBON DIOXIDE, PER CENT			OXYGEN, PER CENT		
	NO. 4 BURETTE	NO. 5 BURETTE	DIFFERENCE	NO. 4 BURETTE	NO. 5 BURETTE	DIFFERENCE
Oxygen Tent	1.59	1.21	0.32	41.21	41.48	0.27
Pneumothorax Air	0.0	0.0		20.96	21.05	0.09
Anesthetic Apparatus	4.31 2.88	4.26 2.80	0.05 0.08			
Rebreathing Experiment into Spirometer	3.68 4.78 3.36 2.93 0.72 1.94	3.94 4.96 3.30 2.67 0.75 1.90	0.26 0.18 0.06 0.26 0.03 0.04			
Tank of Oxygen and Nitrogen				12.54 9.99 12.11	12.23 10.19 12.45	0.31 0.20 0.34

TABLE II

COMPARISON OF SIMPLIFIED PROCEDURE WITH CARPENTER-HALDANE METHOD

ANALYSIS OF AIR COLLECTED FROM CHAMBER IN OPEN CIRCUIT PROCEDURE FOR THE DETERMINATION OF BASAL METABOLISM

CARBON DIOXIDE, PER CENT						OXYGEN, PER CENT					
SIMPLIFIED PROCEDURE				CARPENTER-HALDANE ANALYSIS (5)	DIFFERENCE BETWEEN COLUMNS 4 AND 5	SIMPLIFIED PROCEDURE				CARPENTER-HALDANE ANALYSIS (10)	DIFFERENCE BETWEEN COLUMNS 9 AND 10
NO. 4 BURETTE (1)	NO. 5 BURETTE (2)	DIFFERENCE (3)	MEAN (4)			NO. 4 BURETTE (6)	NO. 5 BURETTE (7)	DIFFERENCE (8)	MEAN (9)		
0.84	0.57	0.27	0.70	0.55	+0.15	20.21	20.66	0.45	20.44	20.36	+0.08
						20.92	20.97	0.05	20.94	20.97	-0.03
0.53	1.00	0.47	0.76	0.55	+0.21	20.53	20.42	0.11	20.48	20.36	+0.12
0.44	0.47	0.03	0.46	0.44	+0.02	20.41	20.92	0.51	20.66	20.46	+0.20
0.50	0.57	0.07	0.54	0.52	+0.02	20.56	20.23	0.33	20.40	20.37	+0.03
0.51	0.64	0.13	0.58	0.52	+0.06	20.06	20.46	0.40	20.26	20.37	-0.11
						20.84	21.05	0.21	20.94	20.97	-0.03
0.62	0.52	0.10	0.57	0.61	-0.04	20.62	20.29	0.33	20.46	20.32	+0.14
						21.26	21.26	0.00	21.26	20.97	+0.29
0.61	0.57	0.04	0.59	0.64	-0.05	20.48	20.73	0.25	20.60	20.28	+0.32
0.59	0.53	0.06	0.56	0.51	+0.05	20.19	20.48	0.29	20.34	20.37	-0.03
0.21	0.21	0.00	0.21	0.46	-0.25	20.33	20.57	0.14	20.45	20.46	-0.01
0.40	0.00	0.40	0.20	0.46	-0.26	20.20	20.60	0.40	20.40	20.46	-0.06
0.56	0.53	0.03	0.54	0.49	+0.05	20.68	20.17	0.51	20.42	20.41	+0.01
0.30	0.24	0.06	0.27	0.39	-0.12	20.22	20.46	0.24	20.34	20.51	-0.17
0.59	0.61	0.02	0.60	0.62	-0.02	20.08	20.22	0.14	20.15	20.28	-0.13
0.42	0.49	0.07	0.46	0.47	-0.01	20.17	20.65	0.48	20.41	20.44	-0.03
0.52	0.55	0.03	0.54	0.54	±0.00	20.42	20.46	0.04	20.44	20.34	+0.10
0.43	0.51	0.08	0.47	0.52	-0.05	20.34	20.49	0.15	20.42	20.37	+0.05
0.42	0.31	0.11	0.36	0.51	-0.15	20.38	20.53	0.15	20.46	20.34	+0.12
0.29	0.48	0.19	0.38	0.44	-0.06	20.29	20.36	0.07	20.32	20.47	-0.15

ADVANTAGES OF THE METHOD

Some of the advantages of the procedure described are listed as follows: (a) The apparatus can be made with ordinary laboratory equipment by any competent glassblower;* (b) the method is suitable for the analysis of gas containing carbon dioxide and oxygen in widely divergent concentrations; (c) regardless of the time elapsed since the last determination, the apparatus is ready for immediate use without the necessity of cleaning capillary tubing or of making a preliminary test for leaks as would be essential with many types of apparatus for gas analysis; (d) only from twenty to twenty-five minutes are required to make duplicate analyses; (e) after relatively little practice accurate results can be obtained by a technician who has had no previous experience in gas analysis.

SUMMARY

1. An apparatus and a method for the determination of carbon dioxide and oxygen, or both, in samples of gas are described.

2. The method is easy to use and duplicate analyses can be made in from twenty to twenty-five minutes.

3. The apparatus is ready for immediate use regardless of the time elapsed since the last determination.

4. The method is very satisfactory for determinations in which an accuracy of 0.3 per cent is sufficient.

5. The apparatus is valuable for making determinations of samples of gas taken from cylinders containing mixtures of oxygen and nitrogen, from anesthetic apparatus, from oxygen tents, or from the thoracic cavity of patients with pneumothorax.

REFERENCES

1. Boothby, Walter M., and Sandiford, Irene: Laboratory Manual of the Technic of Basal Metabolic Rate Determinations, Philadelphia, 1920, W. B. Saunders Company, p. 80.
2. Fieser, Louis F.: A New Absorbent for Oxygen in Gas Analysis, J. Am. Chem. Soc. 46: 2639, 1924.
3. Lewis, Robert C., Kinsman, Gladys M., and Iliff, Alberta: The Basal Metabolism of Normal Boys and Girls From Two to Twelve Years Old, Inclusive, Am. J. Dis. Child. 53: 348, 1937.
4. Carpenter, Thorne M.: An Apparatus for the Exact Analysis of Air in Metabolism Investigations With Respiratory Exchange Chambers, J. Metab. Research 4: 1, 1923.

*If the expense involved is not a matter of consideration, an apparatus with the zero mark of the burette at stopcock *A* may be substituted for the one herein described. The only advantage of such a refinement would be that the total volume could be read directly; the accuracy of the apparatus would be in no way enhanced.

THE ESTIMATION OF THE ENZYMES, AMYLASE, PROTEINASE, AND LIPASE IN DUODENAL CONTENTS*

ALFRED H. FREE, PH.D., AND VICTOR C. MYERS, PH.D., D.SC.
CLEVELAND, OHIO

SOON after the introduction of the duodenal tube as a successful method for obtaining duodenal contents, studies of the enzyme activities of this material were made. A variety of methods utilizing various chemical principles have been employed in such studies. In 1934 a committee appointed by the American Gastroenterological Association[1] conducted a study of the methods used to determine the enzyme activities of duodenal contents. A report of this committee stated: "It is obvious that this survey denoted the existence of unsolved technical difficulties in pancreatic enzyme analysis, otherwise there would be more unanimity of opinion favoring one system of tests. There is insufficient proof at the present time that we have a system of tests which is sufficiently accurate and simple to be routinely adopted for clinical purposes." The clinical usefulness of such enzyme studies has been recently reviewed.[2-4] The present investigation was undertaken to devise a system of enzyme methods that would employ optimal environmental conditions, would be applicable to use in a routine clinical biochemical laboratory, and could be readily evaluated by the clinician.

METHOD OF OBTAINING AND DILUTING DUODENAL CONTENTS

Duodenal contents may be readily obtained by means of a duodenal tube. The authors have made studies of the activities of fasting contents and contents obtained after stimulation of pancreatic secretion with olive oil introduced into the duodenum.[5, 6] Other investigators have utilized fasting contents[7] as well as contents obtained after using a variety of stimulants including magnesium sulfate, dilute hydrochloric acid, peptone solution, corn oil, olive oil, cream, beef bouillon, secretin,[8-12] and mecholyl.[11]

The sample to be analyzed is refrigerated between the time of obtaining from the patient and the time the analyses are to be made. Repeated studies have indicated that there is no significant alteration in enzyme activity of duodenal contents during a period of twenty-four hours, provided the sample is not strongly acid due to contamination with gastric juice. In such cases the results are quite unreliable even though the analyses are made immediately. One c.c. of the sample is made up to a volume of 50 c.c. by adding 10 c.c. of 0.2 molar phosphate buffer (pH 7.2) and distilled water. The 1:50 diluted sample is added to 9.5 c.c. of 0.2 molar phosphate buffer (pH 7.2) to give a

*From the Department of Biochemistry, School of Medicine, Western Reserve University. The data presented in this paper are in part taken from the dissertation submitted by Alfred H. Free to the Graduate School of Western Reserve University, June, 1939, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Received for publication, July 2, 1943.

1:1,000 dilution. This dilution is used for the determination of amylase. It is important to make the dilution with the buffer rather than with distilled water, since marked destruction of amylase may occur when samples are diluted with distilled water.

REAGENTS

1. *General Reagent*

A. 0.2 molar phosphate buffer, pH 7.2, prepared by dissolving 7.62 Gm. of anhydrous potassium dihydrogen phosphate and 20.45 Gm. of anhydrous disodium hydrogen phosphate in water to give a total volume of 1 liter.

2. *Reagents for Amylase*

A. One per cent soluble starch solution, prepared by suspending 1 Gm. of soluble starch (Merck, according to Lintner) in about 5 c.c. of cold water and then pouring about 90 c.c. of boiling water into the suspension. This solution is boiled for exactly one minute and then made up to a volume of 100 c.c. This solution should be refrigerated and should be prepared fresh approximately every two weeks. Even after standing in the refrigerator for a period of about three days the starch solution acquires a turbidity. If the flask containing the solution is heated for a minute in a boiling water bath, however, the turbidity will disappear and the digestibility of the solution will be the same as a freshly prepared solution.

B. Standard glucose solution which is a saturated solution of picric acid containing 1 mg. of glucose in each 3 c.c. of solution.

C. 0.5 molar sodium chloride solution.

D. Saturated solution of sodium carbonate.

3. *Reagents for Proteinase*

A. Five per cent casein solution, prepared by suspending 5 Gm. of casein (Merck, according to Hammarsten) in about 50 c.c. of water and then adding 40 c.c. of 0.1 normal NaOH and sufficient water to make a total of 100 c.c. This solution should be refrigerated and should be prepared fresh about every two weeks.

B. Twenty per cent solution of trichloro-acetic acid.

C. Nessler's solution prepared by method of Folin.[13]

D. Digestion mixture for micro-Kjeldahl determination consisting of 50 per cent sulfuric acid containing 0.25 per cent copper sulfate.

E. 0.3 per cent gum ghatti solution, used for micro-Kjeldahl determination.

F. Nitrogen standard containing 0.283 Gm. of ammonium sulfate per liter (0.3 mg. of nitrogen in 5 c.c.).

G. Standard tyrosine solution containing 0.15 mg. of tyrosine in 3 c.c. Method of preparation described by Anson and Mirsky.[15]

H. Phenol reagent of Folin and Ciocalteau.[14]

I. 0.5 N sodium hydroxide solution.

(Solutions C, D, E, and F are needed only if the micro-Kjeldahl determination of nonprotein nitrogen is employed in the proteinase method.)

4. *Reagents for Lipase*

A. One per cent bile salt solution, prepared by dissolving 1 Gm. of bile salts (Wilson Laboratories, Chicago) in 100 c.c. of water. This solution should be refrigerated, and should be prepared fresh approximately every two weeks.

B. 0.1 normal sodium hydroxide in alcohol, prepared by adding sufficient saturated solution of sodium hydroxide to 95 per cent alcohol to give a 0.1 normal solution. The base is standardized against 0.1 normal acid in the conventional manner.

C. Tributyrin, obtained from Eastman Kodak Co., Rochester, New York.

METHOD FOR DETERMINATION OF AMYLASE

The principle of the method for the determination of amylase is based on the measurement of the amount of reducing sugar formed when a sample of diluted duodenal contents is incubated with a solution of soluble starch. Under proper environmental conditions, the amount of reducing sugar formed is proportional to the amount of amylase present so that this figure furnishes an index of the enzyme concentration.

Add 6 c.c. of 1 per cent soluble starch to a test tube along with 1 c.c. of 0.5 molar sodium chloride and 2 c.c. of phosphate buffer (pH 7.2 to 0.2 M). This mixture is warmed to 38° C. in a constant temperature water bath or a constant temperature oven and then 1 c.c. of the 1:1,000 diluted duodenal contents is added. The tube is thoroughly mixed and replaced in the water bath for exactly thirty minutes. At the end of this time a small amount of dry solid picric acid (about 0.5 Gm.) is added and the contents of the tube saturated with picric acid by continued mixing. This effectively stops enzyme action and provides a saturated picric acid solution for the determination of reducing sugar. Three c.c. of the supernatant solution are pipetted into a Myers-Bailey sugar tube, and 1 c.c. of saturated sodium carbonate solution is added. A standard is prepared by taking 3 c.c. of the glucose standard in picric acid and adding to it 1 c.c. of the saturated sodium carbonate solution. The tubes are placed in a boiling water bath for exactly twenty minutes to effect color development. The standard is diluted to 10 c.c. and the unknown to either 10, 15, or 20 c.c., following which the solutions are compared in a colorimeter. The equivalent of glucose which is produced in the digestion is calculated by the equation $S/R \times D/10 \times 1.0 \times 10/3 =$ mg. of glucose equivalent. A blank which represents the reducing action of the starch must be determined and subtracted from the above value. This will be quite constant for a given batch of starch solution on any particular day and can be readily determined by carrying through the entire procedure described above for the digestion mixture with the exception that the diluted duodenal contents are not added to the reaction mixture until after the precipitation with picric acid. Since all of the reducing value of the blank is contributed by the starch solution, one blank is sufficient for a series of determinations.

METHOD FOR THE DETERMINATION OF PROTEINASE

Proteinase is determined by incubating diluted duodenal contents with a solution of casein and then determining the amount of protein digestion products formed. The index of proteinase concentration is taken as the amount of nonprotein nitrogen formed from the casein or as the amount of material formed which will react with the phenol reagent.

Three c.c. of casein solution are added to a test tube along with 3 c.c. of water and 3 c.c. of phosphate buffer (pH 7.2 to 0.2 M). The test tube is placed in the constant temperature water bath at 38° and allowed to reach equilibrium temperature. To the tube is now added 1 c.c. of 1:50 diluted duodenal contents and the mixture thoroughly stirred. The tube is replaced in the bath and digestion is allowed to proceed for exactly thirty minutes, at the end of which time the tube is removed and 2 c.c. of 20 per cent trichloro-acetic acid are added. This serves to stop the enzyme action as well as to precipitate the undigested casein. The solution is filtered and either a 1 c.c. or a 2 c.c. aliquot is used for a nitrogen determination according to the micro-Kjeldahl procedure described later, or a 1, 2, or 3 c.c. aliquot is used for the colorimetric phenol reaction described later. A choice of either of these methods is offered depending on the facilities of the laboratory, since it has been found as a result of several hundred analyses that the two methods give the same results. It is the authors' belief that if the laboratory routinely carries out determinations of nonprotein nitrogen on blood the micro-Kjeldahl nitrogen estimation might be the method of choice whereas otherwise the colorimetric phenol determination would be more convenient.

For the determination of nonprotein nitrogen the following procedure is employed: An aliquot of filtrate is placed in a large Pyrex test tube calibrated at 35 c.c. and 50 c.c. One cubic centimeter of sulfuric acid digestion mixture is added along with a glass bead, and the mixture is digested over a flame or on a hot plate until perfectly colorless. Two or three drops of superoxol are added at intervals to facilitate the digestion. The digestion material is cooled, some water and 1 c.c. of gum ghatti solution are added, and the solution made up to 35 c.c. A standard is prepared in the following manner: Five c.c. of standard nitrogen solution are measured into a similar tube, 1 c.c. of the digestion mixture added along with 1 c.c. of gum ghatti and diluted to 35 c.c. Fifteen c.c. of dilute Nessler's solution (Folin) are added to both the unknown and the standard, and the colors are compared in a colorimeter. The calculations are based on the formula: $S/R \times 12/A \times 0.3 =$ mg. of nitrogen produced in the digestion. A is the volume of the aliquot used for the nitrogen determination.

Casein solutions prepared according to the above method contain small amounts of nonprotein nitrogenous substances. Correction for this is made by running a blank digestion in exactly the same manner as described above with the exception that the diluted duodenal contents are added after the precipitation with trichloro-acetic acid. This is filtered, and the nitrogen is determined in the filtrate according to the method described. One blank is sufficient for a series of determinations, and this blank value is subtracted from the total nonprotein nitrogen.

If the proteinase activity is to be determined by the colorimetric phenol reaction, the following procedure is employed: Three c.c. of the trichloro-acetic acid filtrate are measured into a test tube, to which are added 6 c.c. of 0.5 N NaOH and 2 c.c. of diluted phenol reagent diluted 1 to 3 with distilled water just before using. A blue color develops spontaneously; this should be compared after ten minutes with a standard solution of tyrosine. The stand-

ard is prepared by treating 3 c.c. of tyrosine standard with sodium hydroxide and phenol reagent in the same manner as the filtrate. Where the amount of proteinase activity is relatively large, the color of the unknown will be considerably darker than that of the standard and will thus result in a certain error due to the disproportionality of the colors. In this case the use of a 1 c.c. aliquot of filtrate is necessary. In order to make the final volume the same, as well as to secure the proper reaction, 2 c.c. of a mixture of phosphate buffer, trichloro-acetic acid, and water in the proportion in which they occur in the filtrate are added to the tube containing the 1 c.c. aliquot. This is then made alkaline, and the color is developed in the usual manner. If this is done, the proportionality of enzyme concentration to the intensity of color developed will hold. Of course, the use of a smaller amount of filtrate must be taken into account in the calculations. The calculation of the amount of proteinase activity may be made by the following formula: $S/R \times 12/A \times 0.15 =$ mg. of tyrosine equivalent produced in the digestion.

A blank determination is necessary, since the casein solution will yield a filtrate after precipitation that contains a small quantity of substances which react with the phenol reagent. The blank is prepared according to the method described above, and the filtrate is then subjected to the same treatment which is given to the digest filtrate. The value of the blank is subtracted from the value obtained in the digestion mixture.

METHOD FOR THE DETERMINATION OF LIPASE

Lipase is determined by incubating duodenal contents with tributyrin and then titrating the amount of acid liberated. The index of lipase concentration is calculated from the amount of acid released.

Into a 200 mm. test tube are measured 4 c.c. of phosphate buffer, 1 c.c. of 1 per cent solution of bile salts, and 5 c.c. of 1:50 diluted duodenal contents. The tube is warmed for three or four minutes in the constant temperature bath at 38° C. One c.c. of tributyrin is then added, and the contents of the tube are thoroughly agitated. The agitation of the tube at this point is important, since it is responsible for the formation of a semiemulsion which is required for the reaction. The tube is replaced in the constant temperature bath and enzyme action is allowed to proceed for one hour, during which time the temperature of the bath is maintained at 38° C. At the end of this time the tube is removed, and 5 c.c. of alcohol and a few drops of phenolphthalein are added. The contents are then immediately titrated with 0.1 N NaOH in 95 per cent alcohol to the first color change of the phenolphthalein.

Since phosphate buffer is present, there will be a small blank titration even though no lipase digestion has occurred. This amounts to approximately 2.75 to 3.00 c.c. of 0.1 N alcoholic NaOH. When this blank is subtracted from the titration, the resulting value gives a measure of the lipase activity. Since the blank is entirely due to the phosphate buffer and tributyrin, one blank will suffice for a series of determinations.

With the environment described for the lipase estimation, the relation between the amount of acid liberated and the amount of enzyme present is not linear. Therefore, it is necessary either to prepare a graph and read the values from the graph or to use a table and obtain the values from the table.

This, although somewhat troublesome, does not impair the accuracy of the final results obtained by this method.

VARIATIONS OF ENZYME ACTIVITY WITH VARYING ENZYME CONCENTRATION

In both the amylase and proteinase method there is a linear relation between the index of enzyme activity and the enzyme concentration over the range of values encountered in normal and abnormal samples of human duodenal contents. This relation is shown in Figs. 1 and 2. Therefore, in the case of amylase determinations, the amount of reducing sugar produced by the digestion can be used as an index of enzyme concentration. Similarly in the proteinase determination the amount of nonprotein nitrogen liberated from the casein or the amount of tyrosine-like material liberated can be used as an index of enzyme concentration. In the lipase method, however, there is not a direct linear relation between the amount of enzyme present and the amount of acid liberated. The curve for the variation of acid liberated with variations in enzyme concentration is shown in Fig. 3. Therefore, in order to secure an accurate index of lipase concentration, it becomes necessary to use the curve or to employ a table such as that indicated by Table I.

STUDIES OF FACTORS INFLUENCING ENZYME ACTIVITY

Many of the steps in carrying out determinations of enzymes are quite arbitrary, since the methods for enzyme measurement are based on determination of enzyme activity. By variation of each of the arbitrary steps in the procedure it is possible to obtain a method which estimates the enzyme under optimal environmental conditions. Fig. 4 shows the effect of variations in pH on the activity of amylase in duodenal contents. It will be seen that when the digestion mixture is buffered with phosphate buffer of pH 7.2, there is an optimal conversion of starch to reducing sugar. Therefore, this pH was employed in the amylase determination. Since optimal digestion by proteinase and lipase also occurs at this reaction, the same buffer is used in the procedure for the estimation of proteinase and lipase. A complete description of the experimental studies that have been carried out in the development of the methods here described has been reported elsewhere.[16] It should be stated that not only is the pH optimal, but also the substrate concentration, buffer concentration, salt concentration, and the amount of duodenal contents employed are optimal for the methods described.

ENZYME ACTIVITIES OF DUODENAL CONTENTS OF NORMAL SUBJECTS

When fractional samples of duodenal contents are obtained after stimulation of pancreatic secretion with olive oil, there is a considerable variation in the enzyme activity of various fractional samples. For this reason the maximum enzyme concentration of the fractional samples has been selected as a more constant criterion of potential enzyme activity.[6] The maximum amylase, proteinase, and lipase values obtained in 17 studies made on 11 normal subjects are shown in the first three columns of Table II. The average maximum enzyme activities for these normal subjects are amylase, 10.99 mg. of glucose equivalent; proteinase, 2.24 mg. of tyrosine equivalent; and lipase, 13.92 c.c. of 0.1 N acid liberated (uncorrected).

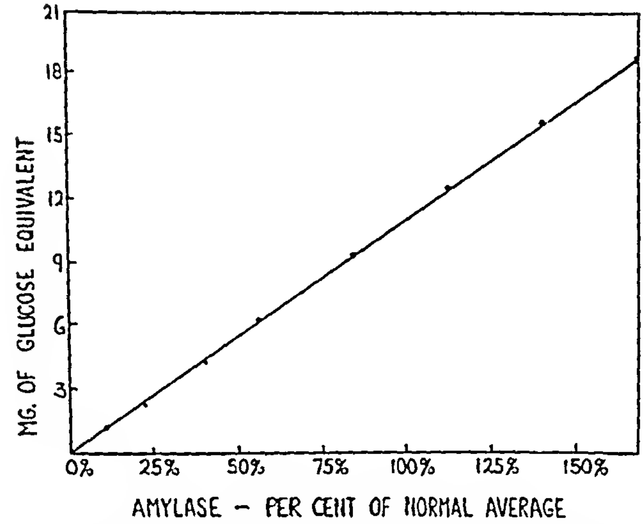


Fig. 1.—Relation of amylase activity to amylase concentration. The variations in enzyme concentration were attained by using different amounts of the same sample of duodenal contents. For convenience the various concentrations are expressed as a per cent of the normal average. Amylase activity is expressed as mg. of glucose equivalent formed in the digestion mixture.

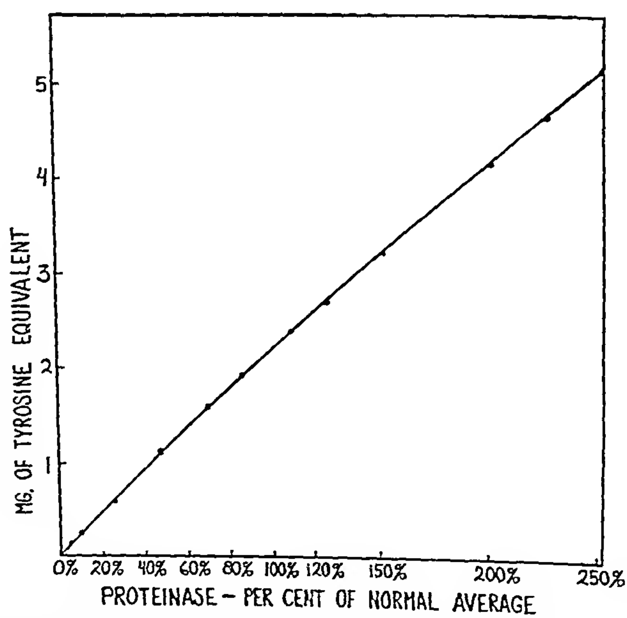


Fig. 2.—Relation of proteinase activity to proteinase concentration. The variations in enzyme concentration were attained by using different amounts of the same sample of duodenal contents. For convenience the various concentrations are expressed as a per cent of the normal average. Proteinase activity is expressed as mg. of tyrosine equivalent formed in the digestion mixture.

The authors have proposed a simplified method of expressing enzyme values.[4-6] According to this system any individual finding is expressed as a percentage of the normal average value. On this basis the values for the normal subjects are shown in the last three columns of Table II. The conversion of amylase or proteinase figures to this system is quite simple and merely involves multiplying by a factor. The factor for the conversion of mg. of glucose equivalent to amylase as a per cent of the normal average is 9.1. For proteinase determined from the tyrosine reaction one needs to multiply the mg. of tyrosine equivalent by the factor 44.4. If the amount of nonprotein nitrogen formed by the proteinase is measured (the average amount of nonprotein nitrogen obtained in the normal subjects is 4.70 mg.), the factor is 21.3. The values for lipase as a per cent of the normal average can be approximated from the data given in Table I or from Fig. 3.

Using this method of comparison the range of variation of amylase is from 55 per cent to 165 per cent of the average normal, whereas proteinase varies from 45 per cent to 148 per cent while lipase varies from 46 per cent to 147 per cent. In the subjects in whom more than one study was made there was considerable variation in the maximum enzyme concentration, but this variation was less than that observed in the different subjects.

This system of expressing results of enzyme studies as a per cent of the normal average can also very readily be applied to results obtained in patients as has been done by the authors.[6] It thus greatly facilitates the clinical interpretation of such data. This method is quite logical, since enzyme measurements determine relative rather than absolute concentrations, the relative concentration being derived from the activity of the enzyme.

DISCUSSION

It should be pointed out that the principles of the methods that are described above are not new. In certain respects the amylase determination makes use of the same principles employed by Myers and Killian[17] in the determination of blood amylase or diastase. Picric acid reduction is particularly well adapted for the measurement of the substances resulting from starch digestion, since maltose, which is the principal product of amylolysis, exerts approximately twice the reducing effect on picric acid that it does on most copper reagents.[18] The use of sodium chloride in the digestion mixture enhances the activity of the amylase and removes the possibility that with some samples the chloride content might influence the measured amylase activity. The use of a pH of 7.2 gives a much greater activity than that obtained at other reactions, such as the pH of 8.4 employed by McClure, Wetmore, and Reynolds.[19] This is clearly demonstrated in Fig. 4.

The proteinase method is in certain respects patterned after the proteinase methods of McClure, Wetmore, and Reynolds,[19] and Anson and Mirsky.[15] The latter investigators employed hemoglobin as a substrate and maintained that this is the only reproducible substrate for proteinase estimations. The reproducibility of casein as employed in the proteinase method has been carefully investigated by means of an experiment in which several different batch preparations of Merck's Casein, according to Hammarsten, were obtained and were used as substrates with the same sample of duodenal contents as a source of enzyme.

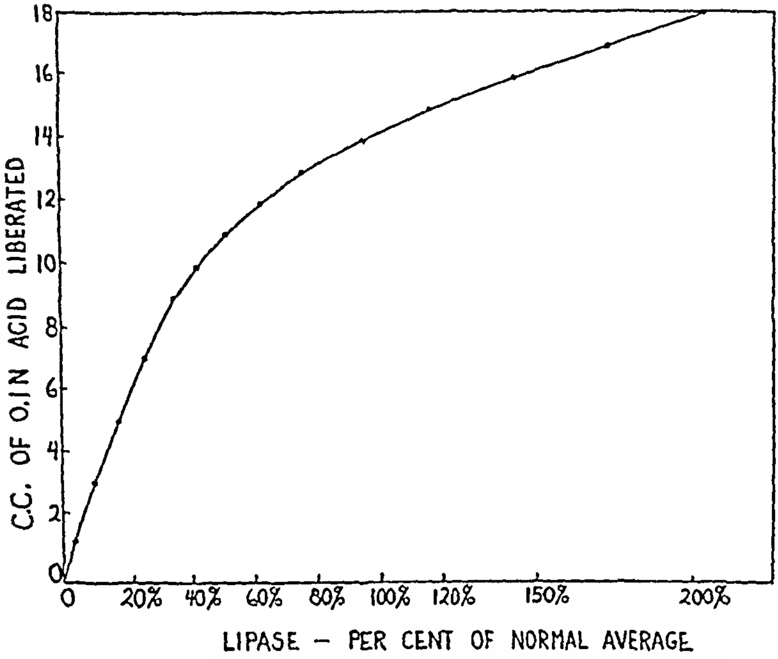


Fig. 3.—Relation of lipase activity to lipase concentration. The variations in enzyme concentration were attained by using different amounts of the same sample of duodenal contents. For convenience the various concentrations are expressed as a per cent of the normal average. Lipase activity is expressed as the c.c. of 0.1N acid liberated in the digestion mixture.

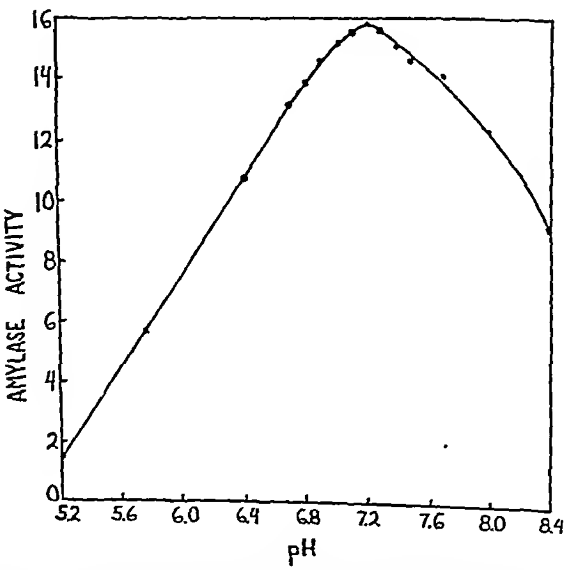


Fig. 4.—Effect of pH on amylase activity. Variations in pH were accomplished by varying the ratio of the acidic and basic salt in the phosphate buffer. All other factors were maintained constant.

The values obtained with the different samples of casein were identical. A sample of casein prepared in the laboratory by a comparable technique also gave a satisfactory result. Experimental data also indicated that a casein solution prepared by the described technique could be used for as long as three weeks without any change in digestibility, although during this time there was some increase in the value of the blank.

TABLE I

RELATION BETWEEN ACID LIBERATED FROM TRIBUTYRIN AND LIPASE ACTIVITY

TITRATION AMOUNT OF 0.1N ACID LIBERATED	AVERAGE NORMAL LIPASE PER CENT
1 c.c.	2.6
3 c.c.	9.0
5 c.c.	17.5
7 c.c.	26
9 c.c.	35
10 c.c.	43
11 c.c.	53
12 c.c.	61
13 c.c.	77
14 c.c.	97
15 c.c.	118
16 c.c.	145
17 c.c.	175
18 c.c.	219
19 c.c.	272

TABLE II

MAXIMUM ENZYME ACTIVITIES OF NORMAL SUBJECTS

SUBJECT	AMYLASE MG. OF GLUCOSE EQUIVALENT	PROTEINASE MG. OF TYROSINE EQUIVALENT	LIPASE C.C. OF 0.1N ACID LIBERATED (UNCORRECTED)	AMYLASE PER CENT OF AVERAGE	PROTEINASE PER CENT OF AVERAGE	LIPASE PER CENT OF AVERAGE
A. H. F.	11.2	1.90	12.50	102	85	70
A. H. F.	14.4	2.29	15.25	131	102	124
A. H. F.	11.5	2.24	14.05	105	100	97
A. H. F.	13.5	2.91	14.20	123	130	101
J. M. C.	7.05	1.68	11.30	64	75	55
J. M. C.	8.36	2.17	11.25	76	97	102
J. M. C.	6.65	2.01	13.25	60	90	83
N. H.	11.9	2.35	11.85	108	105	61
N. H.	9.6	1.70	16.10	87	76	147
W. E. B.	6.03	1.45	12.85	55	65	74
R. K.	18.2	3.12	15.85	165	139	140
D. H. F.	8.82	1.01	10.10	80	45	46
E. W. M.	8.25	2.47	15.95	75	110	145
L. J.	12.4	3.32	15.70	112	148	137
E. J.	17.5	3.30	15.40	158	146	130
H. E.	10.6	2.04	14.40	96	91	104
I. A.	10.8	2.29	13.70	98	102	90
Average	10.99	2.25	13.92	100	100	100

The lipase method employs the same principle that has been made use of in a large number of methods of lipase estimation. In most of these methods the amount of acid liberated by the hydrolysis of a substrate catalyzed by lipase has been measured. Chace and Myers[20] used ethyl butyrate as a substrate; McClure, Wetmore, and Reynolds[19] employed cotton seed oil; Wads-

worth and Aaron[21] utilized triacetin; and Cherry and Crandall[22] used olive oil emulsion. There have been several objections raised to the use of tributyrin[22] as a substrate for lipase estimations, since it has been maintained that the digestion of tributyrin is accomplished by an esterase and not by the lipase involved in the splitting of higher fats. In a series of approximately 500 determinations in various normal and abnormal samples of duodenal contents by the method described above, parallel determinations of lipase by a method quite similar to that described were made in which the substrate was an olive oil emulsion. In every one of the samples studied the amounts of acid liberated from tributyrin and from olive oil emulsion were practically identical. In no instance would the clinical interpretation of the finding be any different whether olive oil or tributyrin was used. Since tributyrin is somewhat more convenient to obtain and handle, the method using tributyrin is preferred by the authors to the method using olive oil emulsion. The addition of bile salts to the digestion mixture is designed to eliminate any effect the amount of bile in a sample of duodenal contents might have on lipase activity.

Intestinal contents obtained by means of a Miller-Abbott tube from an isolated segment of intestine[23] have been studied in an endeavor to ascertain whether intestinal secretions when uncontaminated with pancreatic juice possess any enzymes that will give appreciable enzyme activity under the environmental conditions described in the above methods. Intestinal secretions obtained from two normal human subjects did not show any appreciable enzyme activity by the methods described. This would indicate that alteration in the enzyme concentrations of duodenal contents are entirely due to variations in the enzyme concentration of the pancreatic juice or variations in the amount of pancreatic juice in relation to the total volume of duodenal contents.

There is a considerable range of variation in the enzyme activity of duodenal contents of normal human subjects. This makes the definition of the pathologic values quite difficult. By a combination of factors we believe that we are able to define more accurately the normal variations and thus obtain an earlier recognition of pathologic values. These factors include: (1) the use of sensitive accurate enzyme methods; (2) the elimination of certain variability of pancreatic secretory activity by using the maximum enzyme activity of fractional samples; and (3) the expression of the values by a convenient system which permits evaluation on the basis of the normal average. This has been demonstrated by data on a group of fifteen miscellaneous cases, given in Table V of the paper,[6] presenting observations obtained with these methods.

SUMMARY

Methods are described for the estimation of the enzymes of the duodenal contents, amylase, proteinase, and lipase, in which an optimum pH and optimum concentrations of substrate, buffer, and salt are employed. The values for the enzyme activities are recorded in terms of the normal taken as 100, and thus permit ready interpretation by the clinician.

REFERENCES

1. Aaron, A. H., Bockus, H. L., and Ivy, A. C.: A Report of the Committee "On a Survey of the Enzyme Tests, as Performed by the Members of the American Gastroenterological Association," Am. J. Digest Dis. 1: 256, 1934.

2. McClure, C. W.: Functional Activities of the Pancreas and the Liver, New York, 1937, Medical Authors Publishing Co.
3. Lagerlöf, H.: Pancreatic Function and Pancreatic Disease, New York, 1942, Macmillan Co.
4. Myers, V. C., and Free, A. H.: Clinical Enzyme Studies—Factors Involved, Clinical Usefulness and a Suggested Method for Expressing Enzyme Activities, Am. J. Clin. Path. 13: 42, 1943.
5. Myers, V. C., Free, A. H., and Beams, A. J.: Variations in the Enzymatic Activity of Duodenal Contents, Am. J. Digest. Dis. 5: 461, 1938.
6. Free, A. H., Beams, A. J., and Myers, V. C.: Studies of the Enzyme Activities of Duodenal Contents as a Means of Evaluating Pancreatic Function, Gastroenterology 1: 188, 1943.
7. Comfort, M. W., Parker, R. L., and Osterberg, A. E.: Concentration of Pancreatic Enzymes in the Duodenum of Normal Persons and Persons With Disease of the Upper Part of the Abdomen, Am. J. Digest. Dis. 6: 249, 1939.
8. Voegtlin, W. L., Greengard, H., and Ivy, A. C.: The Response of the Canine and Human Pancreas to Secretin, Am. J. Physiol. 110: 198, 1934.
9. Agren, G., and Lagerlöf, H.: The Pancreatic Secretion in Man After Intravenous Administration of Secretin, Acta med. Scandinav. 90: 1, 1936.
10. Diamond, J. S., and Siegel, S. A.: Secretin Test in the Diagnosis of Pancreatic Diseases With Report of 130 Tests, Am. J. Digest. Dis. 7: 435, 1940.
11. Comfort, M. W., and Osterberg, A. E.: Pancreatic Secretion in Man After Administration of Different Stimulants: A Comparative Study, Am. J. Digest. Dis. 8: 337, 1941.
12. Pollard, H. M., Miller, L., and Brewer, W. A.: A Clinical Study of the Secretin Test, Am. J. Digest. Dis. 9: 68, 1942.
13. Folin, O.: Laboratory Manual of Biological Chemistry, New York, 1934, D. Appleton, p. 337.
14. Folin, O., and Ciocalteau, V.: On Tyrosine and Tryptophane Determinations in Proteins, J. Biol. Chem. 73: 627, 1927.
15. Anson, M. L., and Mirsky, A. E.: The Estimation of Pepsin With Hemoglobin, J. Gen. Physiol. 16: 59, 1932.
16. Free, A. H.: A Study of Enzyme Activities of Duodenal Contents, Cleveland, 1939, Ph.D. Dissertation, Western Reserve University.
17. Myers, V. C., and Killian, J. A.: Studies on Animal Diastases. I. The Increased Diastatic Activity of the Blood in Diabetes and Nephritis, J. Biol. Chem. 29: 179, 1917.
18. Myers, V. C., and Reid, E.: Studies on Animal Diastases. III. A Comparison of Several Methods for the Quantitative Estimation of Diastase in Blood, J. Biol. Chem. 99: 595, 1933.
19. McClure, C. W., Wetmore, A. S., and Reynolds, L.: New Methods for Estimating the Enzymatic Activities of Duodenal Contents of Normal Man, Arch. Int. Med. 27: 706, 1921.
20. Chace, A. F., and Myers, V. C.: The Examination for Diagnostic Purposes of the Enzyme Activity of Duodenal Contents, Arch. Int. Med. 12: 628, 1913.
21. Wadsworth, J. V., and Aaron, A. H.: Observations on Enzymatic Activities of Duodenal Contents, Am. J. Surg. 7: 480, 1929.
22. Cherry, I. S., and Crandall, L. A.: *The Specificity of Pancreatic Lipase: Its Appearance in the Blood After Pancreatic Injury*, Am. J. Physiol. 100: 266, 1932.
23. Abbott, W. O., and Miller, T. G.: Intubation Studies of the Human Small Intestine. III. A Technic for the Collection of Pure Intestinal Secretion and for the Study of Intestinal Absorption, J. A. M. A. 106: 16, 1936.

MEDICAL ILLUSTRATION

NEW IMPROVEMENTS IN MOULAGE PROSTHESIS*

CARL D. CLARKE,† PH.D., WASHINGTON, D. C.

THE word *prosthesis* means an artificial organ, such as an eye, nose, ear, hand, leg, or denture that is used to replace an absent part in the body.

In reprinting one of my articles entitled *Moulage Prosthesis,* the editor of the publication changed the title to *Facial Prosthesis.* This was undoubtedly done for clarification because the word *moulage* is not fully understood. Any author working on the subject of molding and casting is plagued with the necessity for the continued use of these two words. Sometimes he varies them with positive and negative. The word *moulage* has come to be known by a select few in the medical sciences as a wax cast. There are, however, more durable and lifelike mediums than wax. Wax is rapidly becoming a substance for admixing with other ingredients to produce an intermediate stage, pattern, or model in the molding and casting art. Rubber and synthetic plastics, such as *catalin,* are being used for the finished product. To prevent the necessity for repeatedly referring to the two distinct steps, molding and casting, as a unit, the word *moulage* is used to express both. It is of French origin (mouler), meaning to cast or to mold. It should be understood, however, that molding and casting are processes for reproducing an object in form, texture, and often in color, identical in appearance with the original from which the copy or reproduction is made. The mold is the negative; the cast is the positive. The words *pattern* and *model* are used to express the original from which the mold is made. They are used for clarification and amplification, and to prevent repetition and confusion. Since it is possible to make molded and cast prostheses of hands, and casts to cover up scar depressions visible through thin hose on the legs of women, the subject as a whole will be referred to as *moulage prosthesis.* The word *moulage* differentiates the molded and cast prosthesis from the prosthetic appliance which is purely mechanical, such as constructed artificial legs or hands crudely carved from wood and worn with gloves.

Rubber Latex.—The use of rubber latex finally has been established as one of the best materials for the reproduction of artificial parts to be worn on the human body. Synthetic rubber (neoprene) latex is also being employed for this purpose at present. The first mention of rubber latex was made by Clarke[1] and elaborated upon by Bulbulian.[2, 3] In 1936 Mr. Gilbert Blumberg was sent from the Department of Art of the School of Medicine, University of Maryland, to the Mayo Clinic to do moulage work in agar, wax, rubber, and similar

*From the Department of Moulage and Prosthesis, Army Medical Museum.
†Captain, Sanatory Corps, Army of the United States.
Received for publication, April 1, 1943.

materials. Bulbulian evidently used the rubber materials employed there by Blumberg. Further articles by Clarke[4, 5] were written to simplify the technique and to establish a knowledge of the wearing qualities of latex prostheses. Up to this time vulcanite, a hard opaque milled and vulcanized rubber material, was used extensively by dentists for the making of false gums, for holding porcelain teeth, for artificial palates, and similar devices. Kazanjian, Rowe, and Young[6] wrote an interesting article on vulcanite for use in preparing prostheses of the nose and areas around the eyes. Flesh is translucent. This fact can be established by holding the fingers over a burning incandescent electric bulb or flashlight head in a darkened room. Unfortunately, vulcanite is rigid and opaque. It will not move with the face as it assumes various expressions. The prosthesis for the face, if properly made of rubber latex, is soft, flexible, and more fleshlike in appearance. It lends itself more readily to the application of cosmetics and for these reasons is more suitable than vulcanite. Rubber latex can also be made suitably stiff for prosthetic arms, hands, and fingers.

Rubber latex, however, has its own shortcomings and difficulties. Unless properly prepared it darkens on exposure to sunlight. Fugitive or nonpermanent colors used in simulating flesh tones will bleach from the set prosthesis. Shrinkage will occur. Seam lines may appear on the surface of a cast where two or more pieces constitute the mold. All of these disadvantages can be overcome. The methods of doing so will be described. No amount of description or the release of so-called trade secrets, however, will take the place of knowledge gained from the actual working with the necessary materials and from carefully planned and executed workmanship. The information herewith given is substantiated by hundreds of tests and experiments. This approach to the subject is presented because few processes are foolproof. For example, plaster of Paris may be added to water to obtain a homogeneous mixture, but it is more difficult to prepare a smooth mixture if water is added to a quantity of plaster of Paris. Nevertheless, the latter process has been attempted by the novice on numerous occasions. An elementary knowledge of moulage practices is absolutely essential. For this reason *Molding and Casting*[1] is strongly recommended as a reference.

The making of agar molds has been described fully[7] and will not be discussed here. The steps required for producing the prostheses from plaster molds also have been explained.[4] Prostheses or positives of many kinds, however, can be produced from many types of molds, such as those made of rubber, wax, glue, metal, and synthetic plastics.

Because of the demands of World War II the supply of agar has become somewhat limited. Other materials are being substituted for molding and casting with varying degrees of success. An extractive of the seaweed *Chondrus crispus*, commonly known as Irish moss or kelp, is being used in combination with agar in different proportions for the best results. Considerable research should be done on Irish moss to perfect formulas more suitable for moulage purposes.

Another derivative of kelp that is proving more successful than Irish moss is algin or algic acid. It is a protein of marine algae obtained as a by-product

in the preparation of iodine from kelp. Those interested in pursuing this subject should consult American patent No. 2,249,694, "Material for Taking Impressions for Dental and Other Purposes," issued July 15, 1941.

Fig. 1.—A plaster cast is made of the patient.

RUBBER MOLDS AND CASTS

Gum Rubber.—Rubber alone is seldom used for the making of rubber products. It invariably is compounded with other ingredients and these materials greatly influence the properties, quality, and cost of the finished article. It is necessary to mix them with the dried rubber gum by milling, or to the latex or latex paste by agitation. Since this text does not discuss dried rubber, only latex will be considered. The ingredients for mixing with latex are divided into two forms, powders and liquids. Often the powders are converted into liquid or paste mixes for convenience of incorporation; otherwise, the powders may coagulate the latex when stirred into it or the latex will coagulate when stirred into the powder. Of course, this greatly depends on the concentration

of the latex or its dry rubber content, which is spoken of in relation to the *total solid* content of the latex and is an important consideration at all times.

Rubber latex may be procured in two forms,* the unvulcanized latex and the vulcanized latex, often spoken of as prevulcanized latex. The latter form is the simplest to use, but the methods of using both are herewith given.

Fig. 2.—A wax nose is modeled onto the plaster cast or molded and cast from a donor and put in place on the plaster cast.

Unvulcanized Latex.—The unvulcanized or common rubber latex generally requires, in addition to fillers, a vulcanizing ingredient (sulfur), an agent to speed vulcanization (accelerator), a thickening agent (zinc oxide), and a preserving agent (antioxidant). A simple formula for this purpose is:

Rubber, such as latex concentrate	100	parts
Sulfur, flowers of	2	parts

*At present the use of rubber is greatly restricted because of the war. The amount required for prosthetic work is so small and is employed for such an excellent purpose, however, that an application may be made through the Rubber Reserve Board for the small quantities necessary for this work.

Accelerator (Captax)	0.5 parts
Zinc oxide	3 parts
Antioxidant	1 part
Color	q.s.

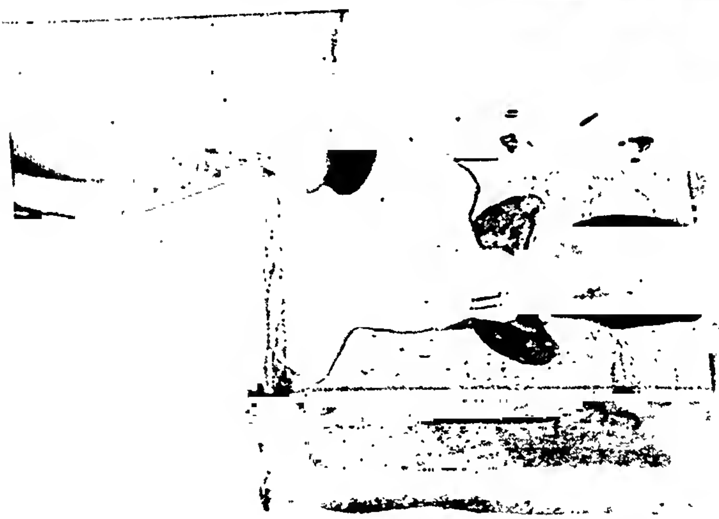


Fig. 3.—A. The wax nose is removed from the plaster cast and painted with a plaster-water mixture on one side only. It is then imbedded into a plaster-water mixture in a box made of glass. Registration notches are cut into the set plaster and a separating medium is applied. A piece of plasteline is put in place to form a pouring funnel in the second half of the plaster mold.
B. The second half of the mold is made.

To this mixture can be added fillers, such as clays, cellucotton, and other ingredients, to increase its bulk and to make it pastelike so that it will remain on a vertical surface to suit specific purposes. A clay filler usually serves best in making a cast; cellucotton or wood flour is more suitable for making molds. Clays are satisfactory for incorporating in latex mixes where the cast is to be poured into plaster molds. White clays and zinc oxide counteract the darkening of set latex when exposed to sunlight.

Unvulcanized or common latex must be vulcanized by heat in one form or another to preserve the rubber. It is done after the rubber has set within the mold and generally before the cast is removed. A rubber mold can be vulcanized on the pattern unless the pattern is made of wax. Unvulcanized latex has a tendency to stick to the pattern or mold unless thoroughly dry and vulcanized; therefore, unvulcanized rubber casts are vulcanized while still in the mold.

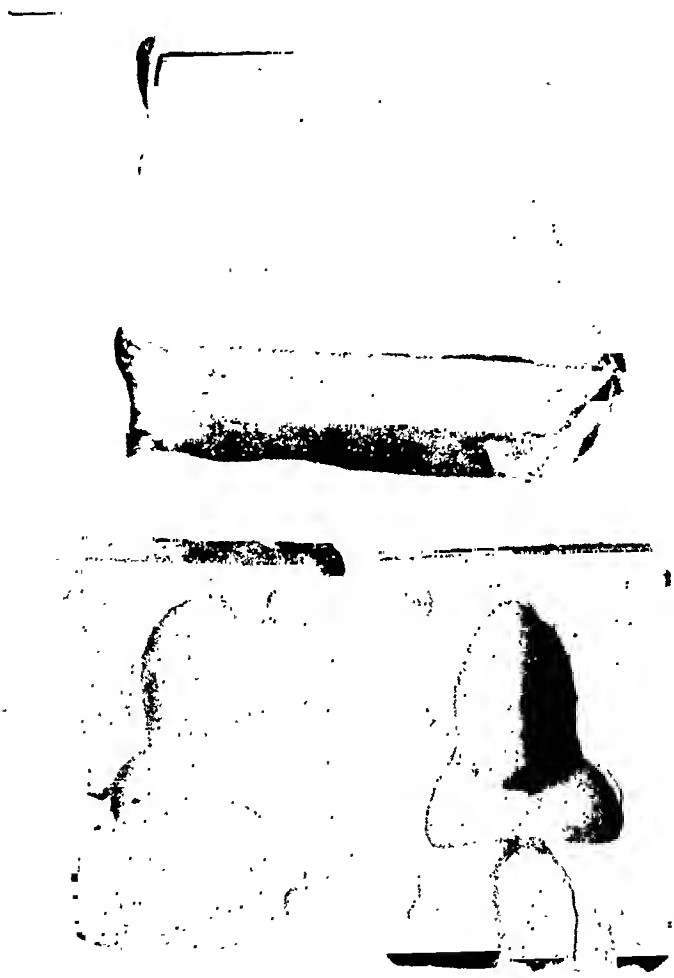


Fig. 4.—*A*. The filled glass box constituting the completed two-piece mold.
B. The mold is opened and the wax cast is removed. If there are undercuts in the cast it may be necessary to destroy the cast to open the mold. In such a case boiling water is poured over the closed mold. The mold will open but the wax cast will be melted.

Vulcanized Latex.—The moulage worker and prosthetist can save considerable time and effort by using vulcanized latex. This is identical in appearance with unvulcanized or common latex; however, it acts in an entirely different manner toward the mold. It will not stick or adhere to plaster and similar patterns or molds when it has set or coagulated. Nevertheless, it should not be removed from the pattern or mold until comparatively dry; otherwise the cast will not retain its shape.

Fillers.—Cellucotton, wood flour, clays, and similar fillers may be added to vulcanized latex in making rubber molds. Since the latex is vulcanized as a liquid before shipment, additional vulcanization is unnecessary. Clays may be added to the prosthetic cast to prevent darkening in sunlight. It should be remembered, however, that the more clay that is added the more translucency is lost. For prosthetic work Bulbulian recommends the addition of 3 Gm. of zinc oxide in a water dispersion to 200 c.c. of vulcanized latex (Vultex H 235). According to tests this will not prevent darkening of the latex. Furthermore, this additional zinc oxide may produce cracking of the resulting prosthesis, since Vultex H 235 already contains some zinc oxide. The cracking, however, may not occur for some months. A white clay filler would be more suitable in this case. The clays do not have the opacity or covering power of zinc oxide; therefore, more than 3 Gm. of clay to 200 c.c. of rubber may be used. This is 1½ per cent. White China clay and Dixie clay suggest themselves for this purpose. Dixie clay, when used in great quantities, not only produces opacity but causes a stiffening of the resulting prosthesis. Titanium dioxide is considered the whitest of the common fillers and possesses a high degree of fastness to light and vulcanization. It is a bright white powder free of yellow tinge; it does not affect the aging of the rubber, but is rather opaque and has considerable covering power. Therefore it must be used in small quantities and should be ground thoroughly in water before use.

The General Latex and Chemical Company* manufactures an excellent clay filler already dispersed in water that is most suitable for prosthetic work. It is their No. H 222, Part B, and can be used in conjunction with Vultex F 293 or H 235. It may be compounded in the Vultex up to 10 per cent without obtaining great opacity or loss of translucency. Of course, this depends on the color and opacity to be produced.

An excess of fillers and sulfur causes rubber to become stiff on drying. A formula for prosthetic work should not include enough of these ingredients to be objectionable. In some cases, however, extreme lightness is desirable. The stiffening of the prosthesis can be offset by pouring a thin rubber coating into the mold, and by then forcing sponge rubber or foamed latex between the thin layers. This produces a lifelike prosthesis that lends itself to trimming of seam lines and yet remains sufficiently flexible to move on the flesh as different expressions are assumed. Sponge rubber alone does not lend itself to trimming and is not as natural in appearance. It does allow the penetration of air through its pores and this has some advantages. Sponge or foamed rubber will be discussed more fully later in this text.

A simple formula containing vulcanized latex is as follows:

Vultex F 293	10 parts by volume
Filler H 222, Part B	1 part by volume
Pontamine fast red 8 B1 (in water)	q.s.

Rubber latex turns slightly yellow on setting and drying. Caucasian skin color generally is obtained by mixing reds and yellows. Since the latex itself is light yellow on drying, only the red is added. Other reds than the Pontamine

*Cambridge, Mass.

fast red mentioned can be used. Colors and coloring of the finished prosthesis will be discussed in detail in a future article. It should be mentioned, however, that the latex stock solution is given a light basic color in the latex mix to resemble a flesh color. This is done with water-soluble dyes having an alkaline reaction and containing no copper. The final coloring is done with oil and alcohol-soluble dyes, not with pigments as is the common belief. Oil-soluble dyes penetrate the dry rubber and blend in a soft homogeneous manner. The dye-colored prosthesis can be washed and scrubbed with a stiff brush immediately after coloring without any loss of color. If pigments were used they would be scrubbed off because they only lie on the surface. Alcohol-soluble dyes do not penetrate the rubber nor do they blend or bleed out into the rubber. When the prosthesis is colored properly, the necessity for the final use of cosmetics becomes lessened or eliminated.

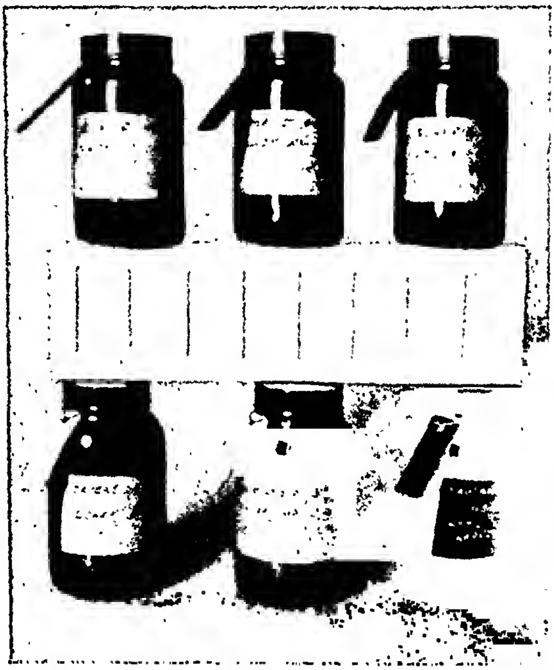


Fig. 5.—Rubber stock solutions of both Caucasian and Negroid skin colors. The plaster block between the bottles is used to obtain dried samples of the various colors. The depressions in this block are numbered from one to eight for specifying the intensity of color.

Pliability.—As already mentioned, the finished prosthesis should possess enough pliability when used as an artificial part on the face to move with the remaining features as they assume various expressions. A thick, solid prosthesis would not have sufficient pliability to move with the features without separating from the skin at corners where the prosthesis joins the face. An example of this inflexibility would be noted in the outer borders of the upper lip. Three alternative remedies for this defect may be considered. The prosthesis can be made rather thin and thus lend itself more readily to shrinkage and distortions, or a thin shell may be filled with sponge rubber to give body to the resulting prosthesis. In the latter instance pliability still remains, in spite of the fact that

the prosthesis seems thick. The third alternative is the use of a softening medium in the latex mix, such as castor oil, mineral oil, and stearic acid. These oils must be converted into emulsions or suspensions in aqueous mediums before being incorporated into the latex mix. Soft soap may be used for this purpose as could any of the substances of a colloidal nature which serve as stabilizers of rubber latex itself, such as casein. Complete information on this subject will be found in most books on rubber latex.

Sponge Rubber.—Prostheses have been made of sponge rubber alone and are rather light and pliable. Air will pass through them, and for this reason it is assumed that they would be more comfortable to wear, since they give the tissues a chance to breathe beneath the artificial organ. In the author's opinion the prosthesis made of sponge rubber is impractical in most cases. It does not lend itself to the trimming of seam lines after removal from the mold. It becomes distorted and remains so. If cosmetics and adhesives are applied to the surface the air cannot pass through the pores. Sponge rubber does not have a natural appearance. Sometimes a comparatively stiff rubber prosthesis is desired, such as a hand, for example. As a matter of fact, a microcrystalline wax is poured into the prostheses of hands to increase the stiffness susufficiently to allow the wearer to hold a pen or pencil for writing or tableware for eating. If the rubber fingers are filled with this plastic wax, they can be moved to adequate positions for holding such implements. The wax core, which in itself is pliable but remains where placed without cracking, is thick enough for this purpose. Microcrystalline waxes will be discussed in detail in another article.

When pliability and lightness are desired in prostheses for parts of the face, both solid and sponge rubbers are used. The technique for producing such a prosthesis is simple: The formula having the correct basic flesh color (monochrome) and other compounding ingredients is poured into the plaster mold and allowed to remain until a deposit or coating of sufficient thickness is formed. This depends on the dryness of the mold, the size of the resulting prosthesis, and the time the mixture remains in the mold. The longer it stays in the mold the thicker the deposit. In small prostheses a coat of sufficient thickness will be built up in a dry mold within three or four minutes or less. The rubber is then poured from the mold and the mold allowed to drain. This can be done by placing the mold on the spout of the bottle from which the rubber was poured. As the mold drains, a small amount of the same formula is put in a dish and agitated by an egg beater until it is well foamed. The rubber should gain at least three times its volume after beating. It will be sluggish and will not pour as readily into the mold. A small amount may be poured into the opening of the mold, however. A large rubber bulb is then used to force this sponge rubber into the mold and the process is repeated until the mold cannot hold more rubber. The mold is then placed in a warm place until the cast or prosthesis is thoroughly dry. This should take place in from twelve to twenty-four hours when the mold is placed in a drying cabinet or on a heated radiator. If the cast is removed too soon it will shrink and become distorted.

Prostheses that are to be enlarged by soaking in benzine[8] for further casting to offset shrinkage should not be cast in sponge rubber, because they disintegrate more quickly in the expanding agent than solid rubber does.

Preparing Sponge Rubber.—Sponge or foamed rubber can be prepared from ordinary latex containing the compounding and vulcanizing ingredients, or from vulcanized latex. An emulsifying agent such as liquid soap is added to

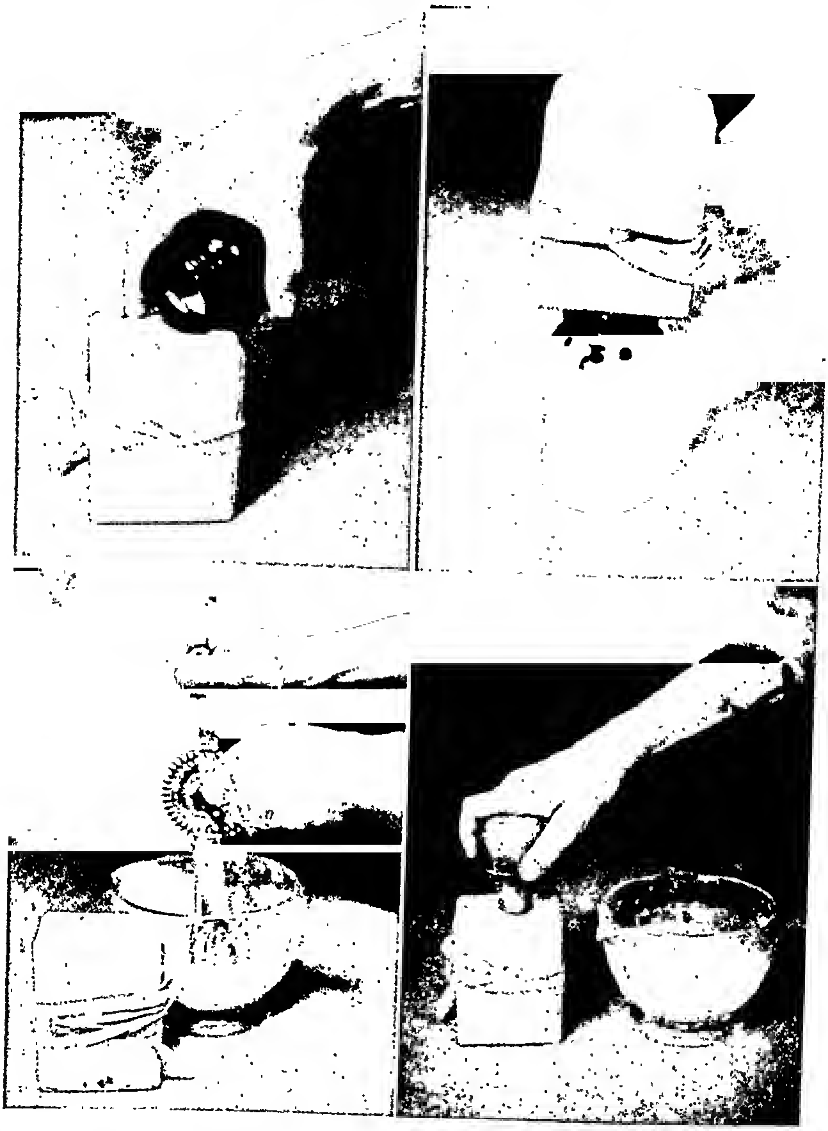


Fig. 6.—*A*. In making a prosthetic rubber nose the cast is poured from the desired stock solution.

B. If it is desirable to use a sponge rubber center for the prosthesis, the rubber is poured from the mold back into the bottle after being in the mold for a minute or more.

C. Part of the rubber from the same stock solution is foamed with an egg beater to form the sponge rubber.

D. Some of this sponge rubber is poured into the mold and forced down with a rubber bulb. From time to time the process must be repeated.

the latex formula in order to stabilize the foam; a setting agent, which is a coagulant having a delayed action, is also added to set the foam. The ingredients are adjusted in the formula, and treatment of the mold is carried out in a manner so that the foam produced by beating, whipping, or blowing the latex mixture is stabilized in this condition. Sodium bicarbonate also may be employed for foaming rubber by effervescence, but this method is not as dependable as

the introduction of air into the latex mix by mechanical means. The soap and salt are added to the mix so that the bubbles will not burst or subside until the setting agent coagulates the whole. The same metallic salts may be used in sponge rubber as in the mixes for coagulating coats or deposits on nonporous molds, such as those of agar, metal, or rubber. After the bubbles and film have set, the rubber is dried. By adjusting the proportion of the setting agent and the temperature of the mold, the time in which the latex froth will set can be controlled readily. Some of the common salts used in sponging latex are calcium sulfate, magnesium sulfate, and calcium chloride. The proportion used is necessarily small, approximately 1/40 part to the latex mix. The salts should be dissolved in water before being added to the latex mix.

SOFTENERS AND PLASTICIZERS

Sometimes it is desirable to have a prosthesis that is solid as well as soft, but made without sponge or foamed rubber. For this purpose softeners or plasticizers are incorporated into the latex mix before it is poured into the mold. These softening agents render the rubber impression more pliable, a desirable feature in facial prostheses which must move with the muscles.

The following are two formulas suitable for this purpose:

No. 1

STEARIC ACID OR PARAFFIN FORMULA

Stearic acid or paraffin	4 Parts
Oleic acid	1 Part
Water	5 Parts
Caustic potash	3 Per cent of weight of stearic acid or paraffin
Ammonia solution	3 Per cent of 26° Be

The stearic acid or paraffin is melted with the oleic acid while stirring vigorously. These ingredients are stirred into the water containing the caustic potash. The ammonia is added while the stirring is continued. The emulsion is carried out best at 70° to 80° C.

No. 2

MINERAL OR PARAFFIN OIL FORMULA

A mineral or paraffin oil emulsion may be prepared by adding 2 to 5 per cent of oleic acid to the oil, and then by rapidly stirring the mixture into water containing 1 per cent of 26 Be ammonia solution. This emulsion may be prepared cold with a concentration of 50 to 60 per cent of oil.

Up to 10 per cent of the paraffin or stearic acid emulsion may be used in the latex mix; but if 2 per cent is exceeded, blooming usually occurs. The mineral oil emulsion does not bloom as readily as the paraffin formula and as much as 10 per cent may be used; however, a staining is more likely to occur with this formula than with paraffin.

For prosthetic work it is advisable to mix stock solutions of one or both of these plasticizers to be incorporated at the time a latex mix is prepared for a specific prosthesis.

Coagulum Deposit.—As previously mentioned, it may be necessary in producing the cast to build up a coagulum deposit on nonporous agar and similar molds to develop sufficient thickness for the cast to be of practical value. The

Fig. 7.—*A*. When the cast is completely dry, the mold is opened and the rubber prosthesis is removed. This illustration shows the front view of the untrimmed prosthesis immediately after removal from the mold.

B. The back view of the same prosthesis showing the area to be joined to the skin.

C. [illegible] a Negro immediately after removal from the mold.

D. [illegible] rubber is poured into the mold and forced down with a rubber [illegible] trimmed [illegible] and smoothed off with benzine, and tinted before being attached to the patient.

coagulating agent can be incorporated into the agar-molding composition, or it may be applied to the surface of the mold. A coagulating agent for mixing with agar compositions is magnesium sulfate (epsom salts), because it has no detrimental effect on the agar. Plaster of Paris or unglazed terra cotta molds need no coagulating agents, since these porous molds absorb the water from the latex

mix and thereby build up a coagulum deposit next to the mold surfaces. Metal molds can be used for rubber casting, since a coagulum deposit can be built up on a metal mold by the application of heat to the mold before the deposit is made. A rubber mold can be used to cast rubber, because a coagulum deposit can be built up on a rubber mold which has been treated previously with a coagulant and a separating medium. A wax mold can be treated with a coagulating agent only, as a separating medium is unnecessary.

A. B.

C. D.

Fig. 8.—The making of a prosthetic rubber hand and arm. *A.* The agar composition is poured into a cloth bag. After it has cooled to near its setting point, the hand and arm are inserted into the bag. The bag is placed in ice water where it remains until the agar has completely set. *B.* Before removing the hand, a slit is made opposite the wrist in the agar mold and cloth bag. The hand is then removed. *C.* The rubber latex composition is poured into the bag where it is allowed to stand until a coagulum deposit of about one eighth of an inch is built up next to the mold. The excess rubber is then poured out. *D.* After the coagulum deposit has fully set, but not dried, a melted microcrystalline flexible wax is poured as a core into the rubber cast while the cast is still in the mold.

Where a thick deposit is necessary it is advisable to use a concentrated latex. The thickness of the deposit is dependent on a number of factors, some of which can be controlled at the compounding stage. If unvulcanized latex is used, the simple addition of the vulcanizing ingredients increases the viscosity of the latex. The addition of 2 or 3 per cent of zinc oxide in particular has been found to have an appreciable thickening effect on latex. If more than 5 per cent is added,

a complete coagulation of the entire mixture may result. Some soluble metallic salts, such as calcium sulfate in small quantities (0.3 per cent of the dry rubber content), cause a slight thickening and render the latex sensitive to local heating. By this addition or pretreating of the latex mix, a firm thick coagulum may be built up when the mixture comes in contact with hot surfaces. Salts of magnesium and zinc also serve for this purpose.

Where a thick coagulum deposit is necessary on nonporous molds, the mold may be dipped, sprayed, or painted with dilute acetic acid. Often this is done after the first thin layer of latex has been deposited. The second layer will then be rather thick. After the deposit has completely coagulated, the excess acid should be washed away with water.

The coagulants commonly employed are acetic acid, formic acid, calcium chloride, acetate, nitrate or formate, zinc chloride, and ammonium acetate in water. Acetone or alcohol solutions are also used, depending upon the molding material and the conditions under which it is desired to effect a coagulation. Methyl alcohol has been of value, because of its readily wetting properties. Two or more coagulants have been used together, as, for example, calcium chloride (2½ per cent) in equal parts of methyl alcohol and water. In fact, I have found calcium chloride and acetic acid among the best coagulants for prosthetic work, according to the problem to be solved. The salt was used in the mix and the acid was applied to the surface of molds. The coagulating agent may be painted or sprayed on the surface of the mold, or it may be poured into closed molds and drained out. It may or may not be dried on the form. A coagulant should be chosen and applied in such a manner, however, that the rubber will be coagulated immediately, so that there will be little tendency for the latex to flow or cause "sags." "Sags" may be the result of two thin coagulated surfaces of latex between which is a deposit of uncoagulated latex. They may also be produced by coagulating one layer over another in the same mold, a practice which is not always good. It is best to try to build up a deposit of sufficient thickness in one pouring. After the deposit is made it must be dried. In the case of prosthetic rubber hands, a melted microcrystalline wax is poured into the hollow hand before it is removed from the mold to prevent shrinkage of the rubber and to make the hand of practical use. In such a case an unvulcanized latex formula may be used, as the hot wax will vulcanize the thin rubber deposit.

STEPS IN MAKING A PROSTHETIC HAND

A practical example of building up a coagulum deposit is obvious when a rubber pattern is to be made from an agar mold for a prosthetic hand and arm. Briefly, the procedure is as follows. A one-piece agar mold is made of the donor's hand and arm by filling a cloth bag with melted agar composition containing epsom salts. The composition should be cooled to a point where it is comfortable to the donor. His hand and arm are inserted into the bag and the bag is placed in a deep bucket of ice water until the agar sets. If started at the right temperature this should take from one-half to three-quarters of an hour. When the agar has set completely the hand is withdrawn. Before withdrawing the hand, however, a slit should be cut on one side of the bag of agar composition opposite the wrist to allow the mold to spread, so that the largest part of and

can pass through the small opening formerly occupied by the wrist. After the hand is removed a gauze bandage is tied around the agar mold at the wrist to close the expansion slit. A coagulating agent is poured into the bag to further treat the surface of the mold. This is drained out and hot air forced in to dry the surface. The mold is then filled with rubber latex which is allowed to stand until a deposit of one-eighth inch is built up on the sides of the agar mold. This deposit is caused by the epsom salts in the agar, plus the surface coagulant. After a one-eighth inch deposit is built up the remaining rubber is poured out

Fig. 9.—The making of a prosthetic rubber hand. *A*. The agar mold is placed in ice water. *B*. The mold is cut opposite the wrist. *C*. The rubber is poured into the mold. After setting, the wax is poured into the cast to form the core. *D*. The cloth bag is removed, and then the agar mold is cut into blocks and broken away from the cast. The rubber hand is still wet and must dry on the wax core. The seam line caused by cutting the agar mold to release the hand is plainly visible on the wrist of the rubber cast. This is trimmed off with hot instruments after the cast has fully dried.

and the mold is placed on its open end over a collecting pan to permit it to drain. When the rubber stops dripping, a hair drier, which is a fan blowing heated air through a small opening, is placed in the open end of the cast to aid in further coagulation of the cast while it is still in the mold. After the rubber cast has coagulated sufficiently to stand handling of the edges, it is filled with a microcrystalline wax to serve as a core to prevent shrinkage of the cast when the agar mold is removed. The rubber at this stage should not be com-

pletely dry, or cracking will result. If the wax is not poured at this time the cast will continue to dry from the inside and considerable shrinkage will take place. The agar mold is removed after the wax has set completely but before the rubber is dry. On removal, it will be found that seam lines were formed on the rubber where the mold was cut opposite the wrist. These are dressed off with heated instruments and all other defects are repaired. Although such a hand can be used as a prosthesis for the patient, it is far better to use it as a pattern for making a one-piece plaster mold so that many duplicates can be made. This is done by applying plaster to the rubber hand to form the mold. After the mold is made, it is placed in an oven and heated until the wax core of the cast is melted. The wax is then poured out. A thin rubber shell of a hand incased in a plaster mold remains. By grasping the rubber edges the collapsible hand may be pulled from the mold. Duplicate hands are then made in the same mold by pouring rubber into it, allowing the deposit to be built up, and pouring out the excess latex. The rubber cast may be dried from within by using the hair drier. The duplicate hand is removed and the process repeated until a sufficient number of prostheses are obtained. The flexible microcrystalline wax is poured into the rubber hands after removal from the plaster mold to give them stiffness and to make them practical for grasping objects. The fingers of the prosthesis are simply bent with the natural hand into the correct position for practical use. The object to be used is then placed into the prosthetic hand with the natural hand.

The prosthetic hand is colored with oil-soluble dyes for softly blended effects and with alcohol-soluble dyes for detail. The oil-soluble dyes bleed into the rubber and permeate the color through the entire thickness of rubber. Alcohol-soluble dyes do not bleed into the rubber but remain where they are placed; therefore, they can easily be washed from the surface. This can be prevented by the application of a fine coat of semimatte lacquer or by the application of a latent rubber solvent to the prosthesis. Such a solvent opens the pores of the rubber temporarily to absorb the dye. The solvent evaporates quickly without causing the surface of the prosthesis to lose detail. Mixtures of turpentine and benzine serve well for this purpose. The turpentine acts slowly on the rubber, whereas the benzine acts rather quickly; therefore, the turpentine is used as a retarding agent for the benzine. Immediately after these materials are applied, the prosthesis can be washed with soap and water to see if the desired effect thus obtained will be permanent.

REFERENCES

1. Clarke, Carl D.: Molding and Casting, Baltimore, 1938, John D. Lucas Co.
2. Bulbulian, A. H.: An Improved Technic for Prosthetic Restoration of Facial Defects by Use of a Latex Compound, Proc. Staff Meet. Mayo Clinic 14: 433, 1939.
3. Bulbulian, A. H.: Prosthetic Restoration of Facial Defects by Use of Latex Compound. A Further Detailed Description of the Technic Used, Proc. Staff Meet. Mayo Clinic 14: 721, 1939.
4. Clarke, Carl D.: Moulage Prosthesis, J. Lab. & Clin. Med. 26: 901, 1941.
5. Clarke, Carl D.: The Application and Wearing of Facial Prostheses, Ibid. 27: 123, 1941.
6. Kazanjian, V. H., Rowe, A. T., and Young, H. A.: Prosthesis of the Mouth and Face, J. Dent. Research 12: 651, 1932.
7. Clarke, Carl D., and Cone, Elizabeth S.: Agar Compositions for Molding, J. Lab. & Clin. Med. 27: 966, 1942.
8. Clarke, Carl D., and Stiner, Frederick M.: The Control of Expansion and Contraction of Rubber Casts, J. Lab. & Clin. Med. 28: 1286, 1943.

The Journal of Laboratory and Clinical Medicine

Vol. 28 | September, 1943 | No. 12

CLINICAL AND EXPERIMENTAL

BLOOD PRESSURE FLUCTUATIONS IN BRONCHIAL ASTHMA*

II. Effect of Epinephrine and Aminophyllin

Howard Osgood, M.D., and Francis E. Ehret, M.D., Buffalo, N. Y.

THE occurrence in bronchial asthma of excessive fluctuations of the systemic systolic blood pressure, synchronous with the respiratory cycle, has been noted in the past by a number of observers, among whom are Tinel and Jacquelin,[1] Rackemann,[2] and Feinberg.[3]

In a recent article[4] one of us drew attention to these fluctuations, and reported observations on a number of asthmatic patients under various circumstances. The conclusions, which agreed closely with those of Tinel and Jacquelin, were: (1) During the asthmatic paroxysm there is constantly present an increase above normal in the respiratory fluctuation of the systolic blood pressure. (2) The amplitude of this fluctuation parallels closely the severity of the asthmatic dyspnea. (3) When the asthmatic paroxysm has ended, the respiratory systolic fluctuation returns to normal limits. An exception to the third statement has been noted: In certain chronic asthmatic patients, in whom irreversible changes have taken place in the lungs, there may be present at times a moderate increase in the respiratory systolic fluctuation, even though the patient appears to be in little or no distress.

Further observations disclosed that the systolic blood pressure uniformly reaches the highest point during expiration and falls to its lowest point during inspiration, both during the paroxysm and during the asthma-free state. Certain experiments with respiratory obstruction in anesthetized cats and in healthy young men, which Osgood has reported,[5] showed a similar increase in the respiratory systolic fluctuation with increasing obstruction. The timing of the high

*From the Medical Services and Allergy Clinic of The Buffalo General Hospital.
Received for publication, December 23, 1942.

and low points of the fluctuation in the respiratory cycle was also the same in these experiments as in bronchial asthma.

The detection and measurement of this respiratory systolic fluctuation are readily made, using an ordinary sphygmomanometer. The method was described in a previous paper.[4] The normal fluctuation in healthy persons with quiet breathing averages about 4 mm. Hg with a range from 2 mm. to 8 mm.

The present paper concerns the effect on this fluctuation of epinephrine and of aminophyllin, administered for the relief of bronchial asthma.

Relief from the dyspnea of bronchial asthma induced by the subcutaneous injection of epinephrine is usually prompt in appearance. It is evidenced subjectively by a rapid diminution of the respiratory distress, and objectively by a decrease or disappearance of the asthmatic wheezes and râles from the lungs: or, if there is much mucus present, by a coarsening of the asthmatic sounds in the chest, and sometimes by the expectoration of secretion. There is also a lessening or a complete cessation of the vigorous muscular efforts of respiration and of the retraction of the suprasternal and supraclavicular tissues.

Following the intravenous administration of aminophyllin with relief, the changes are somewhat different. They are not so marked and are often slower in appearing. They consist subjectively of a feeling of general relaxation and of release from dyspnea, which may be prompt or delayed one-half to one hour, and sometimes of a fleeting feeling of warmth. There may also be a cough with evacuation of sputum. Objectively there is a decrease in the respiratory efforts, as with epinephrine. The retraction of the soft tissues of the chest and the asthmatic sounds in the lungs, however, are apt to diminish to a lesser degree than the subjective relief would seem to indicate. After both drugs it is not difficult to estimate clinically the degree of relief afforded, utilizing these various phenomena.

BLOOD PRESSURE FINDINGS AFTER EPINEPHRINE

Charts I and II present the blood pressure findings in seven patients, all suffering from moderate or severe bronchial asthma at the time. As on all subsequent charts, the high and low points reached by the systolic blood pressure during expiration and inspiration, respectively, are shown in mm. Hg, and the respiratory fluctuation is indicated by the cross-hatching between these points. The degree of dyspnea at the moment of each reading is indicated by the solid black column at the bottom. Below the column is marked the time of the injection of medication and of each reading.

It will be noted that the systolic blood pressure showed wide fluctuations before the epinephrine was given. Within ten to twenty minutes after the injection the systolic fluctuation had returned to within normal limits in those patients who obtained clinical relief.

Patient F. N. (Chart I), suffering from chronic asthma of the "mixed" type, secured prompt relief, and in twenty minutes following the injection of epinephrine the systolic fluctuation had dropped from 14 mm. to 3 mm. On the other hand, M. P. (Chart II), whose chronic asthma was also of the mixed type, obtained practically no relief, and the respiratory systolic fluctuation, which had been 28 mm. beforehand, was still 23 mm. thirty-three minutes after the injection.

The patient C. B. (Chart II) suffered from chronic asthma of the intrinsic type associated with hyperplastic sinusitis. His severe, recurrent attacks were usually relieved temporarily by epinephrine. A reading in May, 1940, during a severe paroxysm, showed a systolic fluctuation with respiration of 24 mm. On August 3, 1940, he was seen in great distress, with his skin gray and sweating. The respiratory systolic fluctuation was 50 mm., one of the greatest we have observed. He was given ½ c.c. of 1 to 1000 epinephrine and 1 c.c. of epinephrine in oil, 1 to 500. He experienced marked relief, and twenty minutes after the injection the systolic fluctuation had dropped to 6 mm. A reading eight months later, when he was in no apparent distress, gave a systolic fluctuation of 12 mm. His lungs, however, showed numerous coarse wheezes throughout.

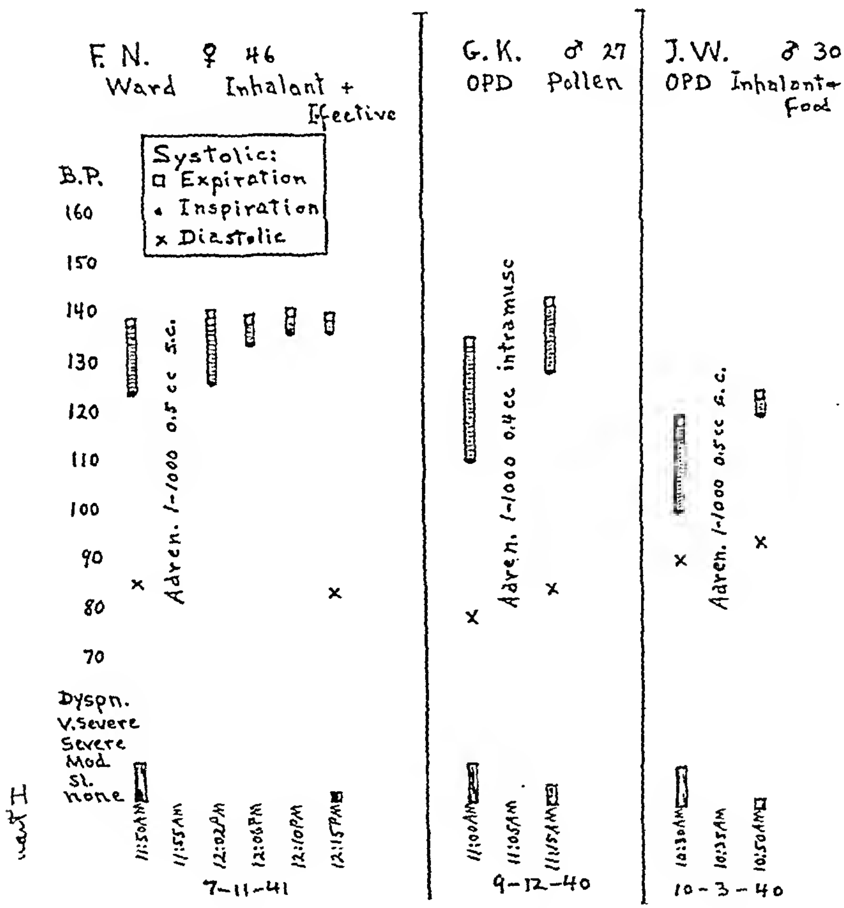


Chart I.—The effect on the respiratory systolic fluctuation of epinephrine administered for the relief of bronchial asthma.

BLOOD PRESSURE FINDINGS AFTER AMINOPHYLLIN

The response of the respiratory systolic fluctuation to the intravenous injection of aminophyllin differs from that of epinephrine. On Charts III and IV are recorded observations on two patients to whom epinephrine and aminophyllin were given at different times for relief of their asthma.

Patient A. B. (Chart III) was sensitive to tree, grass, and weed pollens, and to some other unidentified factors, probably intrinsic. Her paroxysms were periodic and severe, and epinephrine usually gave relief. On April 17th she was seen in a severe attack; her lungs showed many wheezes. The respiratory fluctuation of her systolic pressure was 24 mm. seated, and 38 mm. recumbent. Six-tenths c.c. of 1 to 1000 epinephrine was given subcutaneously, while recumbent. Sixteen minutes later she had no distress and the respiratory systolic fluctuation was 6 mm. recumbent. A week later she was again seen in a moderately severe paroxysm with wheezes throughout her lungs. The respiratory systolic fluctuation was 26 mm. Aminophyllin, 0.24 Gm. in 10 c.c., was given intravenously. Twenty-five minutes later she had experienced considerable subjective relief, although not complete, and her lungs still showed many wheezes. The respiratory systolic fluctuation was still 24 mm.; the expiratory peak of her systolic pressure had fallen from 120 mm. to 110 mm.

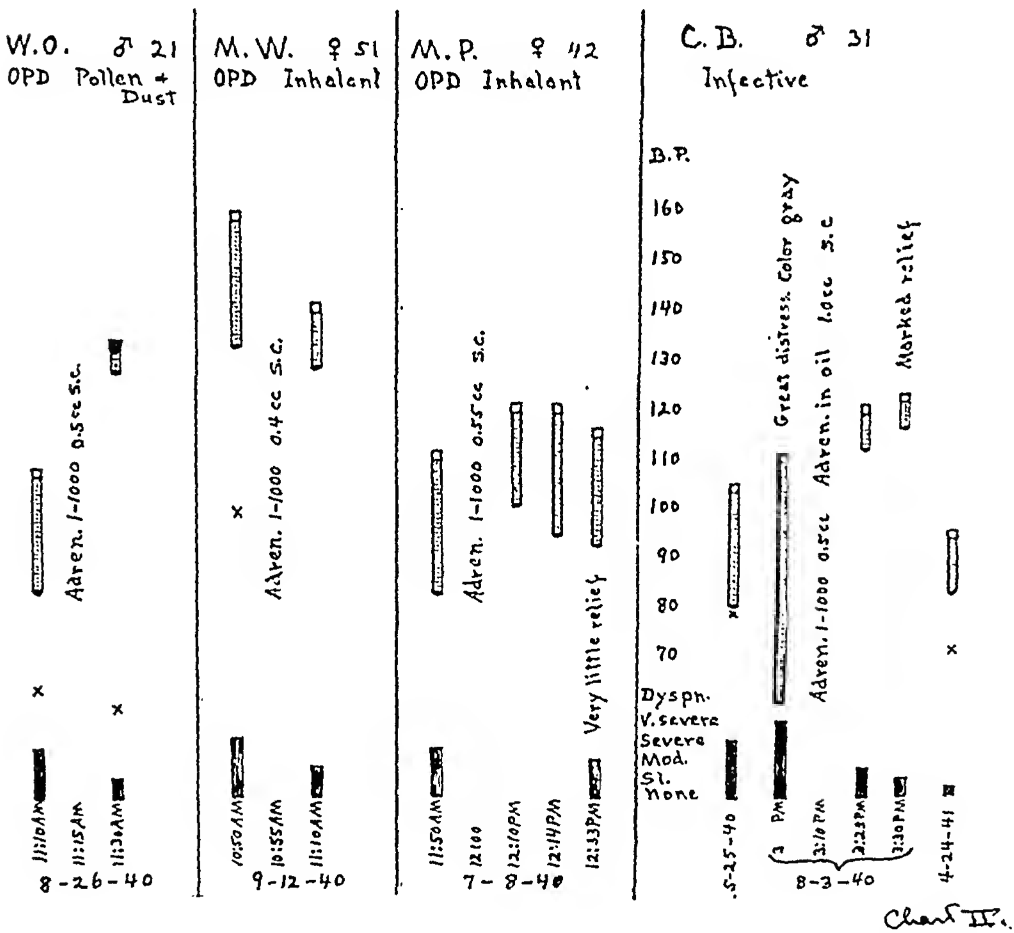


Chart II.—The effect of epinephrine on the respiratory systolic fluctuation in bronchial asthma.

Patient G. H. (Chart IV) was exceedingly sensitive to grass and weed pollens and possibly to molds; her asthma was seasonal. She was seen on July 7 when suffering from severe asthma. The respiratory systolic fluctuation was 18

mm. Epinephrine, 0.8 c.c. in 1 to 1000 solution, was given subcutaneously. Subjective relief was felt in eight minutes, and was marked, though incomplete, in twelve minutes, at which time the respiratory systolic fluctuation was 8 mm. On September 6 she was again having severe asthma. One-half c.c. of 1 to 1000 epinephrine had been injected by her son at 6 A.M. At 7:30 A.M. she was suffering severely, with "tight" wheezes throughout the lungs. The expiratory peak of her systolic pressure was 180 mm. and the respiratory fluctuation was 15 mm. One-half gram of aminophyllin in 20 c.c. was given intravenously. Ten minutes later the expiratory peak of her systolic pressure had fallen to 96 mm.; fifteen minutes after the injection it was 110 mm. She had great subjective relief and began raising considerable sputum. The respiratory systolic fluctuation, however, was still wide: 16 mm. and 20 mm. on two close readings. Twenty-three minutes after the injection, relief was maintained, but the respiratory swing was still 20 mm. Two and a quarter hours later she was somewhat dyspneic with continued expectoration; the respiratory fluctuation was 14 mm. She required no further medication through the day, but was given 1 c.c. of epinephrine in oil at 7:30 P.M. At 10 P.M. she had slight dyspnea and her expiratory systolic peak was 120 mm. with a respiratory fluctuation of 8 mm. The following morning she was asthma-free. The respiratory systolic fluctuation was 8 mm.

Chart V presents the findings in C. G., a married woman aged 29 years, sensitive to pollens and to a number of other inhalants. On October 19 she had been given adrenalin in oil, 1 to 500, at 4 A.M. and at 11 A.M. At 12:30 P.M. she was markedly dyspneic and orthopneic, with retraction of the soft tissues of the chest and sonorous sounds throughout the lungs. The respiratory systolic fluctuation was 40 mm., with the expiratory peak at 140 mm. She was given 0.25 Gm. aminophyllin in 10 c.c. intravenously. Fifteen minutes later she had felt some relief, but the systolic fluctuation was still 40 mm. Relief was progressive, and two hours and forty-five minutes after aminophyllin she was very comfortable, with only slight dyspnea and some wheezes in her lungs. The respiratory fluctuation of her blood pressure, however, was still 26 mm. Two days later she was free of dyspnea and the respiratory fluctuation was 11 mm. and 14 mm. The lungs still contained some sonorous râles.

J. G. (Chart VI), a colored man of 42 years, had long bouts of severe asthma due to intrinsic factors and to foods. He showed at times frequent premature ventricular systoles, although there were no significant electrocardiographic changes or other signs of cardiac disease. His blood Wassermann was negative. Epinephrine afforded so little relief that he refused its administration and asked for aminophyllin. Before injection of the latter drug he would be sitting up, feet over the bedside, wheezing, coughing, and sweating. After its injection he would quiet down in a few minutes and lie semirecumbent, although rarely did his chest clear of wheezes. As will be noted in the chart, the response of the systolic fluctuation to the drug was rather irregular. In general there was a fall in the level of the expiratory peak and a decrease in the respiratory fluctuation, although not so marked as after epinephrine in other patients.

Chart VII gives the blood pressure readings on patient M. M. taken before and after six intravenous injections of aminophyllin and two subcutaneous injections of epinephrine.

She was an unmarried woman, aged 24 years, sensitive to grass and weed pollens, to orris, and to some other inhalants. She had had recurrent attacks of asthma since childhood, which in recent years occurred chiefly in the summer and fall, and lasted several weeks at a time. She had developed surprisingly few permanent changes in the lungs or chest cage. Three years of hyposensitizing treatment had not produced very satisfactory results. Her paroxysms were difficult to control and she was rather "sensitive" to epinephrine. Best relief was secured with aminophyllin, although it was rarely complete and was slow in appearing.

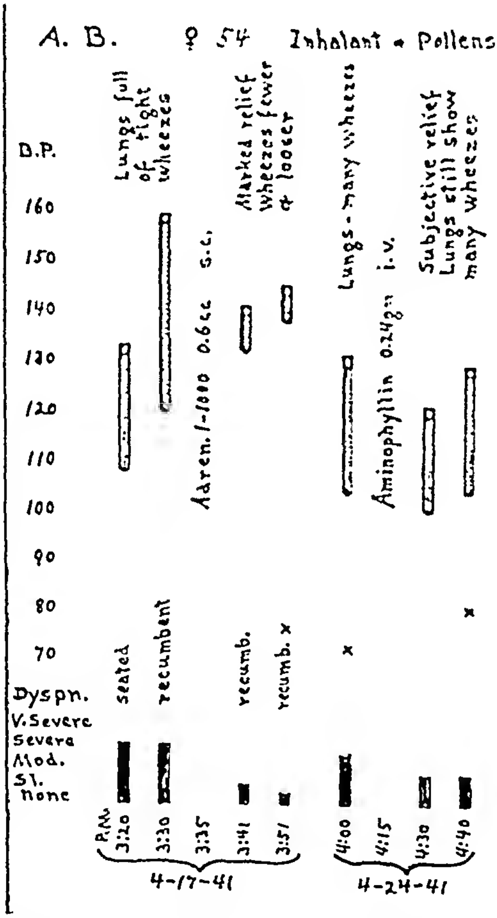


Chart III.—Comparative effects on the respiratory systolic fluctuation of epinephrine and of aminophyllin, administered to a patient in separate paroxysms of asthma.

In her chart it will be noted that with severe dyspnea, the respiratory fluctuation of her systolic blood pressure was wide, varying between 22 mm. and 40 mm. before either drug had been given. After the two epinephrine injections (July 3, 1939, and June 13, 1940) she obtained little or no relief, and the respiratory systolic fluctuation did not decrease in amplitude; the expiratory peak rose slightly.

The six injections of aminophyllin afforded considerable though incomplete relief. She felt "more relaxed" afterwards. Following three of the injections,

the respiratory systolic fluctuation decreased slightly (by 10 mm., 16 mm., and 14 mm. respectively), but did not return even to near normal limits during the period of observation. After the other three injections there was a slight decrease in the fluctuations (6 mm., zero, and 2 mm.). With aminophyllin, such relief as occurred seemed to follow more a moderate fall of the expiratory peak of the systolic pressure than a decrease in the respiratory fluctuation.

Two months later, after she had been asthma-free for five weeks, the respiratory systolic fluctuation was 8 mm.

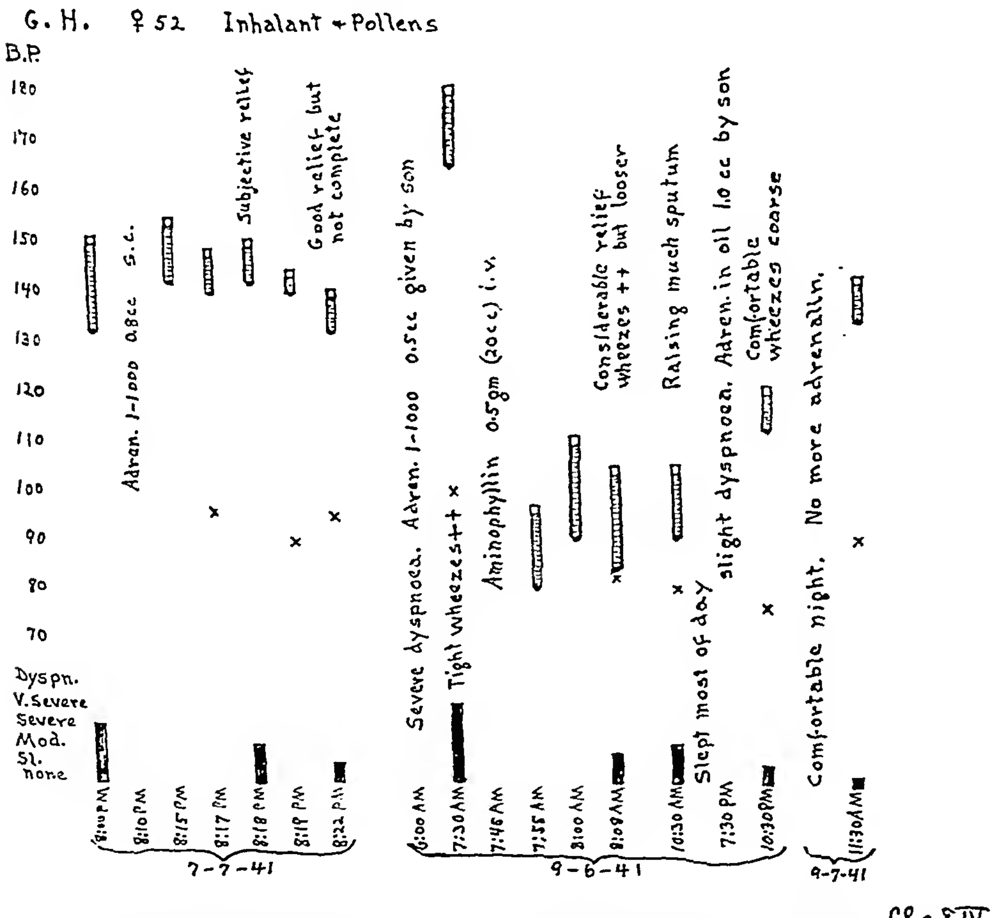


Chart IV.—Comparative effects on the respiratory systolic fluctuation of epinephrine and of aminophyllin, administered to a patient in separate paroxysms of asthma.

DISCUSSION

As a result of observations reported by Osgood in two previous articles,[4, 5] he believes that the enhanced respiratory fluctuation of the systolic blood pressure found in bronchial asthma is dependent upon and is an indirect measure of the obstruction to respiration and that a return of this fluctuation to normal signals a reduction of the obstruction. Consequently the observed return of this fluctuation to normal, when relief follows epinephrine, would indicate that the relief with this drug is brought about by a direct release either of bronchial

spasm, or of bronchial edema, or of both. When relief is obtained with aminophyllin, judging from the fact that the respiratory systolic fluctuation decreases but does not return entirely to normal limits, we may conclude that there has been clinical evidence of *some* bronchodilatation with this drug also. That the fluctuation does not decrease appreciably, either after epinephrine or aminophyllin, when no relief is secured, indicates that the bronchial obstruction has not been released.

Since relief with aminophyllin does not appear to depend wholly on bronchodilatation, the question arises as to what mechanism is here involved.

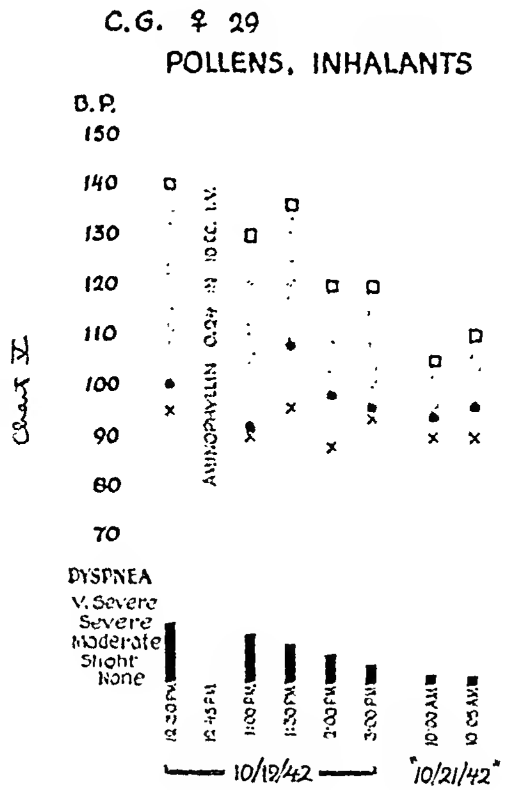


Chart V.—The effect of aminophyllin on the respiratory systolic fluctuation when relief is secured.

Those patients who were given aminophyllin were in general suffering from more obstinate asthma than those given epinephrine, and many were "epinephrine-fast." It is possible that in some of these patients retained bronchial secretion tended to maintain partial obstruction, thus continuing the wide respiratory fluctuation of the systolic blood pressure. This would not, however, explain the rather uniform persistence of a wide fluctuation after aminophyllin with relief.

If the asthmatic patient continued normal respiratory movements during a paroxysm of bronchial obstruction, there would be insufficient ventilation to eliminate properly CO_2 or to take in O_2; hypercapnia and anoxia would result. Leaving out of consideration the possibility that nerve impulses from the lungs, initiated by the respiratory obstruction itself, induce dyspnea, it can be assumed that the asthmatic dyspnea is a reflex response of the respiratory centers, de-

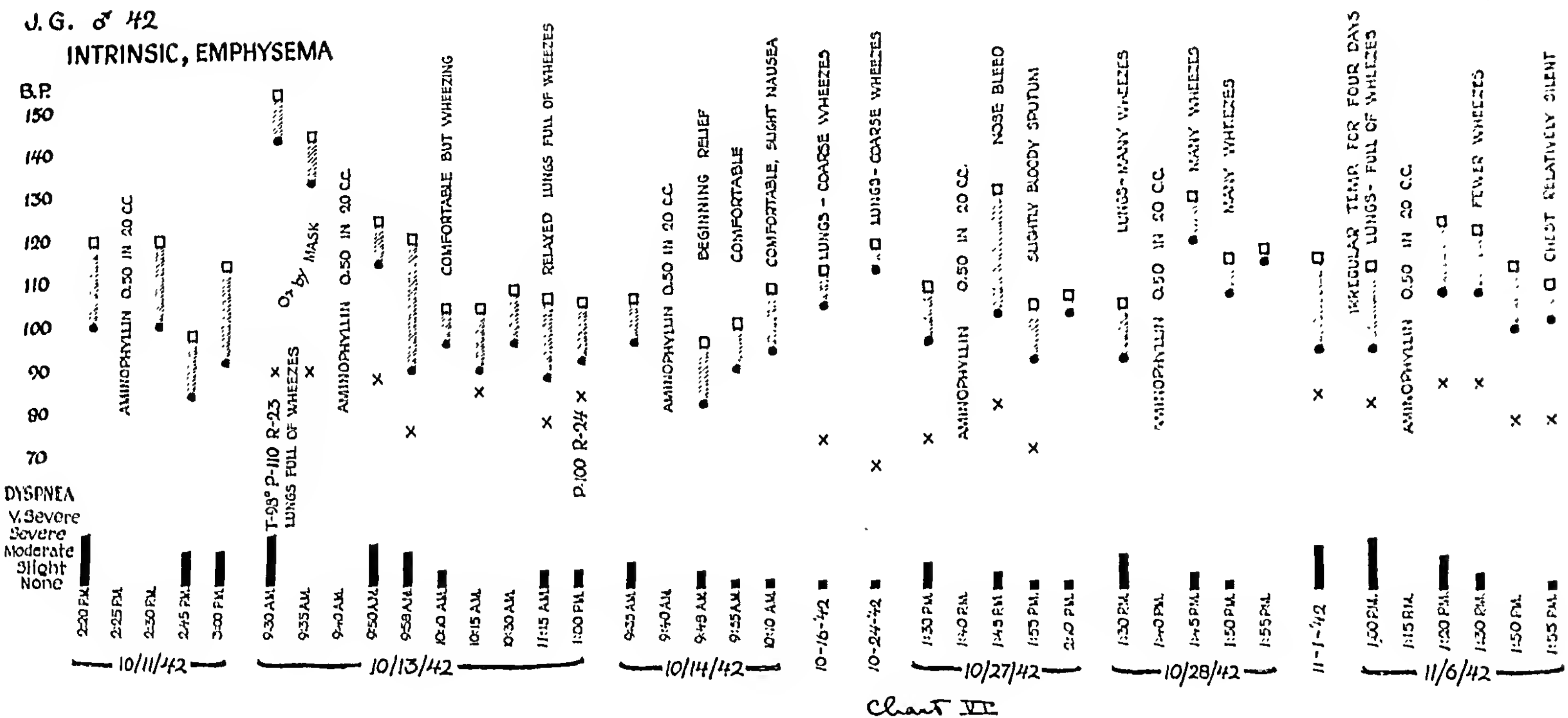


Chart VI.—The effect of aminophyllin on the respiratory systolic fluctuation with variable relief.

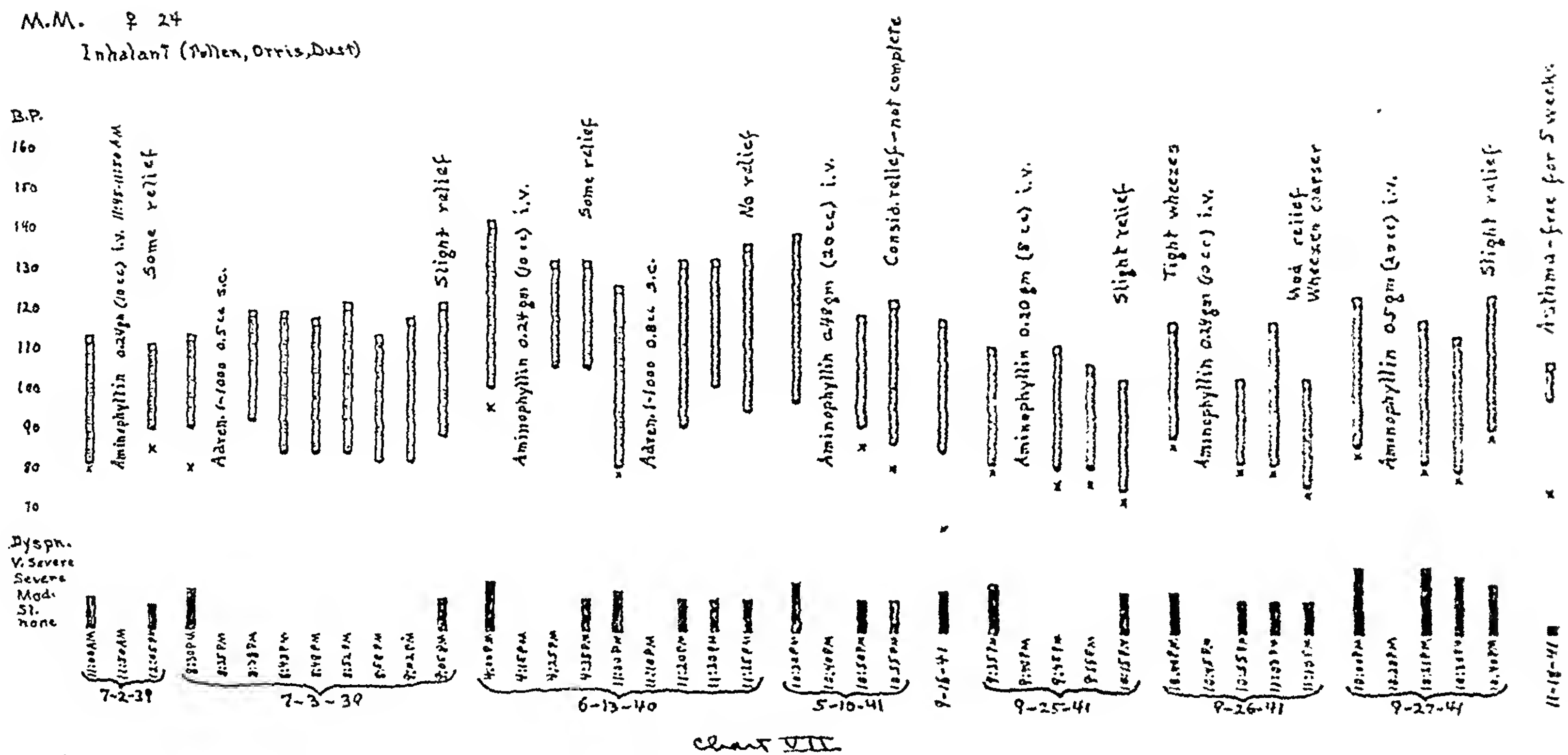


Chart VII.—The effects of epinephrine and of aminophyllin on the respiratory systolic fluctuation in an asthmatic patient refractory to treatment.

pendent primarily upon the CO_2 content of the blood and secondarily on O_2 saturation. If oxygen is administered, the patient will experience some subjective relief even though the bronchial obstruction is not released. The asthmatic type of breathing persists, because if it did not, CO_2 would accumulate and soon stimulate the respiratory centers to further activity. Aminophyllin is said to produce vasodilatation of the coronary arteries (F. M. Smith, et al.,[6] Essex, et al.[7]) and of the renal arteries (Deschamps[8]) and to increase the total blood flow through the pulmonary circulation (Mitchell[9]). The last action would expose more blood per minute to the alveolar membrane and so promote more rapid elimination of CO_2 and greater oxygenation of the blood. The respiratory centers would be subjected to the lesser stimulus of a lower blood CO_2 content; the anoxia would be reduced. The patient would then experience an appreciable diminution of his dyspnea, even though the bronchial obstruction was only partially released.

An alternative supposition, from a purely speculative point of view, is that part of the physiologic response in human beings which eventuates in bronchial asthma may be a pulmonary arteriolar spasm, similar except for reversibility to that occurring in rabbit anaphylaxis. The obliteration of this arteriolar spasm by the vasodilating action of aminophyllin would then explain in part the relief of asthma by this drug. This hypothesis invites further study.

In either case, an improved blood flow throughout the pulmonary circuit appears to us to be an important, if not the principal, factor in the relief of asthma by aminophyllin.

Our interpretation of the data presented here is highly theoretical and open to discussion. It raises a number of questions. Does the venous pressure fluctuate with respiration in asthma, and if so, what time relation does its fluctuation bear to the arterial fluctuation, and how is it affected by epinephrine and aminophyllin? What are the variations of the intrathoracic pressures in asthmatic patients as related to the respiratory systolic fluctuation, and to relief? What changes occur in the blood CO_2 and the arterial O_2 saturation, and in the pulmonary circulation time before and after these drugs? These are answerable only by investigation. It is hoped that others will make additional observations along these lines, testing our findings and carrying them further.

CONCLUSIONS

Following the subcutaneous injection of epinephrine, if relief from asthma is obtained, the respiratory systolic fluctuation decreases to or near the normal.

Following the intravenous injection of aminophyllin, if relief is obtained, the respiratory systolic fluctuation may decrease, but not to an extent comparable to that with epinephrine.

Following either drug, if relief is not secured, the respiratory systolic fluctuation remains wide.

It is suggested that the principal action of aminophyllin in relieving asthma is by increasing the blood flow through the pulmonary circulation by vasodilatation and that its bronchodilating effect is of secondary importance.

A generous supply of aminophyllin was furnished us by G. D. Searle & Co. for use in our studies.

REFERENCES

1. Tinel, J., and Jacquelin, A.: Les modifications circulatoires peripheriques au cours de l'accès d'asthme, Bull. et mém. Soc. méd. d. hôp. de Paris 47: 440, 1931.
2. Rackemann, F. M.: Clinical Allergy, New York, 1931, The Macmillan Co., p. 431.
3. Feinberg, S. M.: Allergy in General Practice, Philadelphia, 1934, Lea & Febiger, p. 99.
4. Osgood, H.: Blood Pressure Fluctuations in Bronchial Asthma. 1. Clinical Observations, J. Lab. & Clin. Med. 28: 927, 1943.
5. Idem: Blood Pressure Fluctuations in Respiratory Obstruction. Experimental Observations, J. Lab. & Clin. Med. 27: 1536, 1942.
6. Smith, F. M., Miller, G. H., and Graber, V. C.: Effect of Caffein Sodio-benzoate, Theobromin Sodiosalicylate, Theophyllin and Euphyllin on the Coronary Flow and Cardiac Action of the Rabbit, J. Clin. Investigation 2: 157, 1925.
7. Wégria, R., Essex, H. E., Herrick, J. F., and Mann, F. C.: Simultaneous Action of Certain Drugs on the Blood Pressure and on the Flow in the Right and Left Coronary Arteries, Am. Heart J. 20: 557, 1940.
8. Deschamps, P. N.: L'Aminophylline, Presse méd. 46: 211, 1938.
9. Mitchell, W. F.: Symposium on Asthmatic State; Aminophyllin, Its Use, Limitations and Manner of Action, Rev. M. Progr., Ohio State University, Columbus, 1940, pp. 25-26.

POLLEN SURVEYS IN THE UNITED STATES: A CRITICAL REVIEW*

Philip M. Gottlieb, M.D., and Erich Urbach, M.D., Philadelphia, Pa.

CAREFUL etiologic diagnosis and specific therapy of hay fever presuppose an accurate knowledge of the pollen in the air in the patients' vicinity capable of producing pollinosis, and of the seasons during which the pollen is present. Although in a given community a relatively few plants are responsible for the vast majority of pollen sensitivities, in the remaining cases it is precisely due to the pollens less common, and hence more difficult to appraise, that such information has particular value, since it makes possible the selection of the proper pollens to be used in testing. Moreover, it is a well-known fact that only a small minority of patients are hypersensitive to but one pollen. And even those physicians who elect to use an extract of only one species of pollen for the treatment of a case with multiple hypersensitivities do not deny the necessity for thorough etiologic diagnosis. Furthermore, it is often desirable to compare the symptoms of both treated and untreated patients, particularly those who fail to react to tests despite evidence of clinical hypersensitiveness, or those who react in the absence of such evidence, with available phenologic information.

For all these reasons, the accumulation of dependable data concerning the distribution and pollination times of plants causing pollinosis is a fundamental step in the clinical management of hay fever. Since the pioneer studies of Scheppegrell and Durham, a considerable store of information has been amassed. Yet, for a territory as extensive as continental United States, there are still many gaps in our knowledge. We are aware, of course, that many unpublished ob-

*From the Allergy Service of the Jewish Hospital, Philadelphia.

Received for publication, December 29, 1942.

servations have been made, but these need not concern us here. It is the purpose of this paper to evaluate the work which has been reported and on this basis to point out how and where future studies may be performed so as to have the greatest value.

The authors undertook to summarize the published data on this subject. In all, the literature yielded 166 studies of varying scope and content. A complete list of the references classified by states and including those for Canada will be found in a recent article.[1] Pollen data may be gathered in two ways: botanical or field observations, and atmospheric pollen counts. Of the recorded investigations, over one-half are based on the former approach, about one-third on the latter, and only about a score on a combination of both.

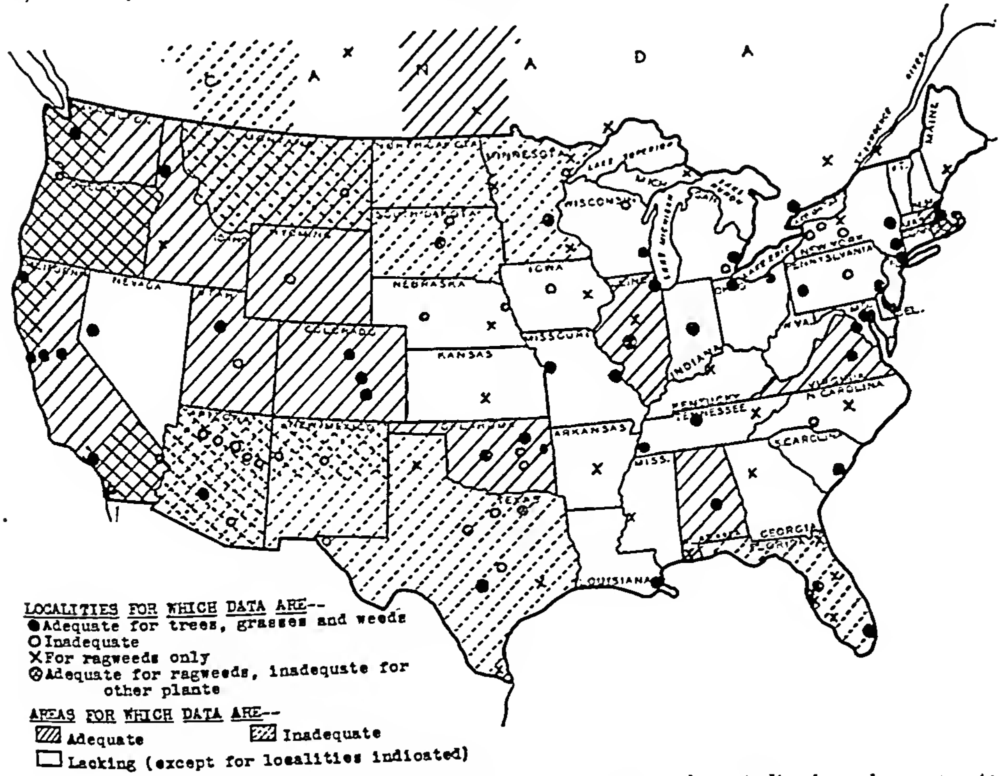


Fig. 1.—Spot map showing the localities and areas for which pollen studies have been reported. A second survey in the same region is indicated by cross-hatching.

GEOGRAPHIC CONSIDERATIONS

Pollen surveys have been reported from most sections of the United States and from some portions of Canada. Most of them concern only one city, but some include observations regarding one species made in a number of cities. Several encompass an entire state or a considerable portion of one. And a few cover several states in extent. Fig. 1 gives the exact locations and regions where such studies have been made. They are classified as inadequate if only one or a few species are considered, if over-all seasons of groups of plants are given instead of individual dates, if no dates are included, or if only a portion of the hay fever season is reported. Because so much work has been done on them

alone, the ragweed observations are indicated separately. It will be seen that, for some reason, the western portion of the country has been covered more intensively than the eastern.

The following cities, each with a population exceeding 100,000 (according to the 1940 census) have never been the scene of a pollen survey: Birmingham, Ala.; Bridgeport, Hartford, and New Haven, Conn.; Wilmington, Del.; Fort Wayne, Gary, and South Bend, Ind.; Des Moines, Iowa; Kansas City, Kan.; Fall River, Lowell, New Bedford, Somerville, and Springfield, Mass.; Flint, and Grand Rapids, Mich.; Camden, Elizabeth, Jersey City, Newark, Paterson, and Trenton, N. J.; Utica, N. Y.; Akron, Canton, Cincinnati, Columbus, Dayton, and Youngstown, Ohio; Erie, Reading, and Scranton, Pa.; Chattanooga, Tenn.; Norfolk, Va., and Tacoma, Wash. In addition, the following cities of the same size have been covered only for ragweeds: Jacksonville, Fla.; Peoria, Ill.; Wichita, Kan.; Syracuse, N. Y.; Houston, Tex.; and Spokane, Wash.

At no place in the following six states,* with a combined population of nearly nine million, have any pollination studies been carried out: Connecticut, Delaware, New Hampshire, New Jersey, Vermont, and West Virginia. If the ragweeds are disregarded, the following eight states, containing over 17½ million persons, may be added to the list: Arkansas, Georgia, Kansas, Kentucky, Maine, Mississippi, South Carolina, and Wisconsin.

Figs. 2 and 3 show directly for the ragweeds and other plants for which states information is not available or not adequate. In the evaluation here the data were considered inadequate for a state if only one city within its borders was studied, or if there was only an incomplete statewide survey. If there were studies from two or more widely separated cities within a state, or if one state wide and one city report existed, the "adequate" classification was assigned. If still more information was found, the data were listed as relatively complete.

If separate maps had been prepared for trees, grasses, and weeds other than ragweeds, the appearance of Fig. 3 would have been modified somewhat, though in the main the changes would not have been great. Table I gives the number of states falling into each subdivision for each of the major groups of plants. It will be seen that the ragweeds are by far the most widely covered, with trees the least. In addition to the states listed above, there is no information relative to the pollination dates of trees anywhere in Louisiana, Nebraska, North Dakota, Pennsylvania, and South Dakota; of grasses in Iowa, Nebraska, and North Carolina; and of weeds other than ragweeds in North Carolina.

BOTANIC CONSIDERATIONS

The reports examined varied greatly in the number of species or groups of plants included. Many involve only one, the greatest emphasis being on the ragweeds. Others consider the entire hay fever season, naming from a few to over a hundred different plants. In all, 863 species in 312 different genera are mentioned in the literature (Table II). Needless to say, not all of them bear equal weight, and in fact 392 species are referred to only once each (6 among the ragweeds, 123 among the other weeds, 169 among the grasses, and 94 among the

*We are fully aware of the fallacy of applying state borders to pollen surveys. Since a number of reports are statewide in scope, however, it forms a convenient, and indeed the only comparable, basis of comparison.

trees). These are presumably unimportant plants nationally, in relation to hay fever. As might be expected, in proportion to the number of genera or species, the ragweeds have received by far the most attention. The amount of consideration given to the other plants is surprisingly uniform. It may be noted that 5,210 pollination dates are included in 166 articles, or an average of about 31 for each. In general, one report contained the dates for a number of species in one locality, or for one species in many places. Some, of course, included many more; others only a few.

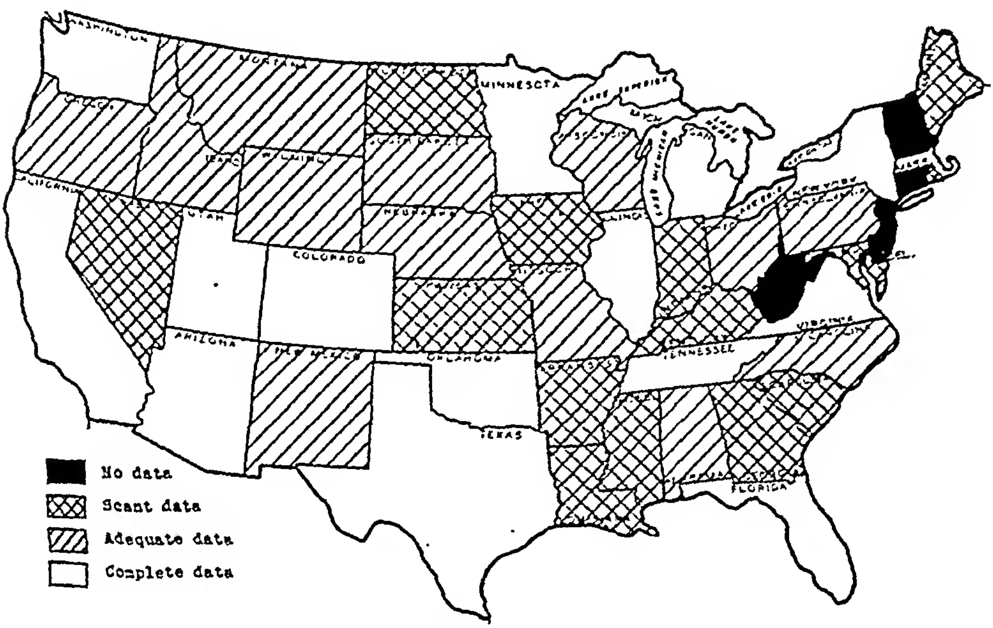


Fig. 2.—Survey of the accumulated data concerning the distribution and pollination times of the members of the ragweed family (Ambrosiaceae) by states.

TABLE I

NUMBER OF STATES WITH AND WITHOUT DATA FOR EACH GROUP OF HAY FEVER-PRODUCING PLANTS

NUMBER OF STATES	RAGWEEDS	OTHER WEEDS	GRASSES	TREES
Entirely Without Data	6	15	17	19
With Scant Data	14	14	14	9
With Adequate Data	13	9	8	10
With Fairly Complete Data	15	10	9	10

TABLE II

AVAILABLE POLLINATION DATA IN THE UNITED STATES FOR EACH GROUP OF HAY FEVER-PRODUCING PLANTS

	NUMBER OF GENERA	NUMBER OF SPECIES	NUMBER OF TIMES MENTIONED	AVERAGE NUMBER OF TIMES MENTIONED PER SPECIES
Ragweeds	4	30	748	24.9
Other Weeds	122	267	1493	5.6
Grasses	108	325	1671	5.1
Trees	78	241	1298	5.4
Total	312	863	5210	5.9

We do not for a moment maintain, either here or in the succeeding two tables, that the number of times a plant family, genus, or species is mentioned need necessarily be an index of its importance in pollinosis locally or nationally. It will be seen, however, that there is likely to be some correlation. Moreover, it is thought that these summarized statistics might be of interest in showing where the attention of the workers in this field is directed.

Table III contains a list of the most important genera, the number of species of each considered, and the total number of times each is referred to in the literature.

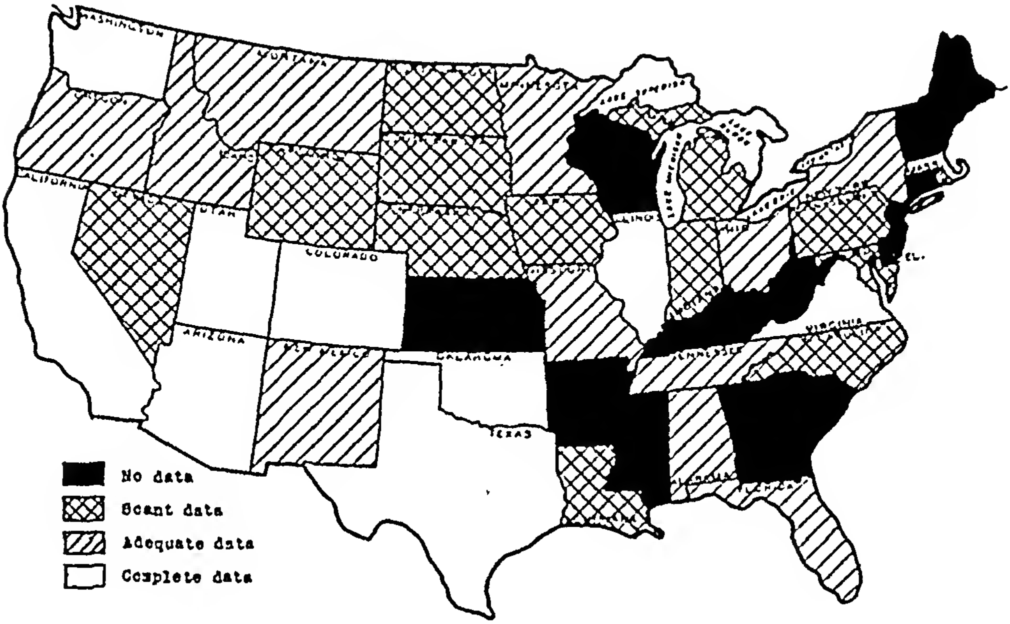


Fig. 3.—Survey of the accumulated data concerning the distribution and pollination times of trees, grasses, and weeds not members of the ragweed family, by states.

Table IV lists the individual species named most often. While notations of the "ragweeds" without differentiation of the specific representatives by far exceed all the rest, it is interesting to note that three grasses and two weeds surpass the leading ragweed. The sum of the figures for the three ragweeds included, however, reaches an impressive total. It will be seen that only four trees are to be found in the table. This is merely a reflection of the fact that many studies give dates only for the genera of trees rather than for each type, and that the statements in others are divided rather uniformly between a number of trees in each genus. Hence, few individual species were able to accumulate any considerable total.

Finally, it not infrequently occurs that a comparison of the pollination dates reported by two or more investigators for the same place reveals considerable discrepancies. For instance, the season for elm (*Ulmus*) in San Francisco is given by Rowe[2] as January and February, and by Deamer and McMinn[3] as the second week in March to the end of April; for orchard grass (*Dactylis glomerata*) in Richmond, Virginia, by Vaughan, Graham, and Crockett[4] as February 23 to June 14, by Blanton[5a] as June 6 to June 23, and by Vaughan[5b] as June

and July; and for giant ragweed (*Ambrosia trifida*) in San Antonio, Texas, by French[6] as July 29 to mid-December (first frost), and by Kahn[7] as mid-September to the end of November. Many other similar examples could be cited. These discrepancies may be explained by differences in method, by the fact that the studies were conducted in different years, or by other factors. In many instances, however, there appears no direct statement as to the techniques employed; the year in which the work was done; whether the season observed is to be taken as typical for the locality; and other pertinent information that is essential if adequate comparisons are to be made between surveys by different investigators in the same area. Variations in the stated prevalence of plants are as great or greater.

It is beyond the scope of the present paper to discuss in detail the techniques for determining pollination data. An evaluation of present knowledge, however, requires some consideration of the methods by which they are obtained. Since certain unavoidable sources of error inherent in these procedures are discussed elsewhere,[1] they need only be enumerated here. In brief, they include variations from year to year in the pollination dates of a given species, and in the relative abundance, pollinating capacity, and clinical importance of different plants, depending on meteorologic conditions; effects of the local growth of cultivated or uncultivated species over a small area; unusual conditions of exposure; and the influence of altitude on the pollination dates and nature of the flora even within a comparatimely limited region. In addition to these factors, however, there are in many studies certain deficiencies which could be rather easily controlled by a careful application of, or slight modification of, the method employed. The nature of these shortcomings and the means by which they might be overcome may best be discussed separately for each type of investigation.

BOTANICAL SURVEYS

In the first place, casual botanical surveys are of little value, since the errors of random sampling may easily affect the findings to a great degree. Only when the region under consideration is painstakingly analyzed from the botanical standpoint and an adequate number of "stations" designated, will the results be significant. Then, each station must be frequently and regularly visited, and detailed notations made of the relative frequency of each anemophilous species, the date of onset and termination of pollination, and the abundance of pollen produced. To insure that the precise dates of each important species can be recorded, the observations must be made at sufficiently short intervals. The average figures for all the stations may be considered characteristic for the area studied. Examples of investigations so performed include, among others, the surveys of Chicago by Koessler and Durham,[8] of the eastern shore of San Francisco by Rowe,[9] and of Winslow and Holbrook, Arizona, by Lamson and Watry.[10]

In this connection, it may be noted that there is no scientific justification for assuming, as many authors do, that the findings in one community are necessarily representative for the surrounding region, the state, or a considerable section of the country, unless such a conclusion is supported by observations throughout the designated area. Thus, Lamson and Watry[11] showed that four

towns in northern Arizona, although only from 42 to 66 miles apart, had very few species of plants in common. Comparable observations have been made by Sellers[12] in Texas, Mullin[13] in Colorado, and by others elsewhere.

TABLE III

GENERA MOST FREQUENTLY MENTIONED IN THE LITERATURE

GENUS	NUMBER OF SPECIES	NUMBER OF TIMES MENTIONED
"Ragweeds":		
Ragweed (Ambrosia)	7	479
Marsh Elder, Poverty Weed (Iva)	8	114
Cocklebur (Xanthium)	7	83
False Ragweed (Franseria)	8	71
Other Weeds:		
Sagebrush, Wormwood (Artemisia)	27	244
Goosefoot (Chenopodium)	16	160
Pigweed (Amaranthus)	9	149
Saltbush, Orache (Atriplex)	27	140
Dock, Sorrel (Rumex)	15	137
Plantain (Plantago)	9	122
Russian Thistle (Salsola)	1	84
Burning Bush (Kochia)	4	48
Water Hemp (Acnida)	2	25
Nettle (Urtica)	6	22
Sunflower (Helianthus)	1	21
Dandelion (Taraxacum)	2	20
Goldenrod (Solidago)	4	19
Hemp (Cannabis)	1	19
Greasewood (Sarcobatus)	1	18
Winter Fat (Eurotia)	1	17
Grasses:		
June, Blue (Poa)	10	185
Brome, Chess (Bromus)	20	105
Wheat Grass, Quack (Agropyron)	12	89
Timothy (Phleum)	1	84
Red Top, Bent (Agrostis)	9	79
Orchard (Dactylis)	2	72
Fescue (Festuca)	13	60
Wild Rye (Elymus)	8	55
Wild Barley, Foxtail (Hordeum)	8	53
Rye Grass (Lolium)	4	53
Bermuda (Cynodon)	1	52
Johnson (Holcus)	4	43
Barnyard (Echinochloa)	4	33
Oats (Avena)	3	29
Salt Grass, Spike (Distichlis)	2	29
Panic Grass (Panicum)	17	27
Sweet Vernal (Anthoxanthum)	1	26
Smut Grass, Rush Grass (Sporobolus)	6	24
Foxtail (Chaetochloa)	5	24
Corn (Zea)	1	24
Koeler's (Koeleria)	1	23
Trees:		
Oak (Quercus)	31	163
Poplar, Cottonwood (Populus)	26	140
Maple, Box Elder (Acer)	10	109
Elm (Ulmus)	8	86
Willow (Salix)	20	68
Birch (Betula)	10	64
Juniper, Cedar (Juniperus)	8	62
Ash (Fraxinus)	11	59
Pine (Pinus)	11	59
Hickory, Pecan (Carya)	8	54
Walnut (Juglans)	5	54
Alder (Alnus)	6	42
Sycamore (Platanus)	4	36
Hazel (Corylus)	3	27

Certain deficiencies encountered in field surveys could be readily avoided. Many reports identify plants only by common names, which are often ambiguous and overlapping. Use of the technical nomenclature is far more precise and has the additional advantage of indicating botanical relationships. Some studies group considerable numbers of plants into rough seasons, such as "spring" or "fall," rather than giving the separate dates for each; others specify pollination times only by months, although greater accuracy would be desirable. The amount of consideration, if any, given to the prevalence of different plants varies tremendously, as does the explanation of the procedures by which the data are gathered.

Reference to herbarium specimens to determine the distribution and pollination times of plants, while it was of use in the early period of pollen studies, is obviously inadequate and requires no further discussion.

But even when most carefully performed and recorded, the botanical survey by its very nature must still be a comparatively crude method, particularly in estimates of the prevalence of the plants and the abundance of pollination. And even when only anemophilous species are considered, it gives no information as to how far the pollens are carried by the wind and whether they ever reach the vicinity of any considerable number of people.

TABLE IV

SPECIES MOST FREQUENTLY MENTIONED IN THE LITERATURE

SPECIES	NUMBER OF TIMES MENTIONED
"Ragweeds" (species not identified)	288
Russian Thistle (*Salsola pestifer*)	84
June Grass (*Poa pratensis*)	84
Timothy (*Phleum pratense*)	84
Lamb's Quarters (*Chenopodium album*)	70
Orchard Grass (*Dactylis glomerata*)	68
Dwarf or Short Ragweed (*Ambrosia elatior*)	66
English Plantain (*Plantago lanceolata*)	61
Red Top (*Agrostis alba*)	59
Red Root Pigweed (*Amaranthus retroflexus*)	59
Giant or Tall Ragweed (*Ambrosia trifida*)	57
Bermuda Grass (*Cynodon dactylon*)	52
Burweed Marsh Elder (*Iva xanthifolia*)	49
Western Ragweed (*Ambrosia psilostachya*)	48
Annual Blue or Low Spear Grass (*Poa annua*)	40
Sheep Sorrel (*Rumex acetosella*)	39
Box Elder (*Acer negundo*)	38
English or Perennial Ray (Rye) Grass (*Lolium perenne*)	38
Curly or Sour Dock (*Rumex crispus*)	38
Johnson Grass (*Holcus halepensis*)	34
Canada Blue Grass (*Poa compressa*)	32
Quack or Couch Grass (*Agropyron repens*)	32
Burning Bush, Firebush (*Kochia scoparia*)	30
Small Poverty Weed (*Iva axillaris*)	29
Sagebrush (*Artemisia tridentata*)	28
False Western Ragweed (*Franseria acanthicarpa*)	28
Meadow Fescue (*Festuca elatior*)	28
Mugwort, Wormwood (*Artemisia vulgaris*)	27
Common Plantain (*Plantago major*)	27
Salt Grass (*Distichlis spicata*)	26
Sweet Vernal Grass (*Anthoxanthum odoratum*)	26
Barnyard Grass (*Echinochloa Crus-galli*)	25
Spearscale, Hastate Atriplex (*Atriplex hastata*)	24
Corn (*Zea mays*)	24
Cocklebur (*Xanthium canadense*)	22
Foxtail Grass, Wild Barley (*Hordeum jubatum*)	21
Giant Wild Rye Grass (*Elymus condensatus*)	21
White Elm (*Ulmus americana*)	20
Giant Poverty Weed, Rough Marsh Elder (*Iva ciliata*)	19
Hemp (*Cannabis sativa*)	19
Pasture Sagebrush, Mountain Sage (*Artemisia frigida*)	18
Black Walnut (*Juglans nigra*)	18
Cottonwood, Carolina Poplar (*Populus deltoides*)	17

POLLEN COUNTS

The pollen count or the direct determination and identification of the pollens in the air, then, is free from these two objections, since it is a quasi-quantitative measure of the actual quantity of the allergen (assuming each pollen grain of the same species to contain an equal amount) in the immediate proximity of one patient or a given population group, depending on the place of exposure. The

simplest technique merely allows the air-borne pollens to fall by gravity to the surface of a microscopic slide coated with a viscous substance, and the number caught on a fixed area (e.g., one square centimeter, one square inch, one square foot, or 1.8 square centimeters) during a unit time, usually one day, is counted and calculated. At the same time, they may be identified within the limits of the botanical differentiation of pollens by comparing them with their known characteristics[14-19] or with a reference collection of the different kinds. Needless to say, such "differential counting" of the pollens yields far more useful data than are obtained from total counts.

The morphology of the grains will vary depending on the adhesive (petrolatum, glycerin, glycerin jelly, corn, linseed, or cedar oil[20]) selected to apply to the slides. Likewise, stains (Lugol's solution, methyl green, Caberla's solution, aqueous or basic fuchsin, aqueous eosin, or nigrosin) or a contrast color method[21] may be used, if desired, to modify the microscopic appearance. Such variations in technique are at the choice of the investigator, who should, of course, be familiar with their effects. They may, in some instances, facilitate the identification, but it should be recognized that they do not in any way affect the actual findings nor lead to more minute differentiation. Recently, specially prepared slides have been suggested[22, 23] to speed the counting and to ensure accurate measurement of the surface area covered.

Various types of apparatus[24-28] have been advocated to obviate the effects of gravity. It is of interest to note that Blackley himself over sixty years ago devised an impingement method. While they have the advantage of permitting the collection of the pollen to be completed in a short period of time and thereby of detecting rapid fluctuations in the degree of atmospheric pollen pollution, they are too cumbersome for widespread practical application and provide no more information utilizable clinically than do the usual techniques. Studies based on slides exposed during airplane flights[29-32, 26] have increased our knowledge of the behavior of pollens in the upper air, but are likewise not clinically applicable.

Probably the greatest single disadvantage of the pollen-counting method is the inability to differentiate between related types of pollen grains. For example, the pollens of all the grasses are identical or nearly so. Likewise, all the members of the family Ambrosiaceae, including the various ragweeds, false ragweeds, marsh elders, and cockleburs must perforce be grouped together, as do those of the order Chenopodiales, containing the pigweed (Amaranthaceae) and goosefoot (Chenopodiaceae) families, since they are microscopically indistinguishable. This constitutes, of course, a serious obstacle to precise work in this field. It may be hoped that by means of differential stains or other techniques, a method will be developed in the future to obviate this difficulty. The pollens of the various genera of trees, on the other hand, are morphologically distinctive.

Most pollen counts have been performed in cities. Just as with field surveys, it is necessary for best results to utilize a number of carefully chosen stations, since differences of terrain, elevation, the presence of tall structures, variations in the flora of the outskirts and in prevailing winds will greatly affect the findings. Such multiple station pollen counts may be exemplified (the list being

by no means complete) by the studies of Koessler and Durham[5] in Chicago, Rowe[6] in the San Francisco Bay area, Acquarone and Gay[33] and Patterson and Gay[34] in Baltimore, and Deamer and McMinn[7] in San Francisco. There is little to be gained by emphasizing the differences between closely situated stations, but the figures for all of them may well be averaged for the community.

The intensive pollen studies by O. C. Durham in conjunction with the Weather Bureau over a number of years in many of our large cities have resulted in the accumulation of a considerable fund of information. Unfortunately, they have practically all been confined to the "ragweeds," including all members of the family, without consideration of other plants. While ragweed unquestionably is our most important plant causing pollinosis, only a small minority of fall hay fever victims are hypersensitive to its pollen alone; most of them are also allergic to other species. Moreover, in the interests of scientific accuracy, it is highly desirable to break down the family into its individual representatives in order to correlate the patients' symptoms with the pollen content of the air, to test adequately, and to treat specifically.

The authors have observed that the figures for ragweed as issued by the Weather Bureau often appear lower than the symptoms of the sufferers would seem to warrant. This is presumably due to the fact that the slides are exposed at the top of a tall building in the center of a built-up metropolitan area, an ideal place for meteorologic observations, rather than at the ground level (or more precisely, the "breathing level") in the residential sections, where the counts may be assumed to be considerably higher because of the effects of gravity and the proximity to fields and vacant lots. This illustrates the importance of choosing the sites of exposure with care, and it may be advisable, as well, to include neighboring suburban areas for similar reasons. In short, it would be advantageous to place the slides where the majority of the population spend the bulk of their day.

It has been customary in most of the reports to give graphs or tables of the daily fluctuations of the pollen counts. While this is informative, clinically it is only of minor interest in the individual case, since every sufferer and every physician is already well aware that there will be variations in the severity of the symptoms from day to day, and indeed, from hour to hour. Moreover, because the curves vary so greatly from year to year (for example, see Durham[35]), the experience of previous seasons cannot be expected to hold in the future. Hence, these quantitative considerations are of little value as compared with the simple knowledge of the species of pollen present and the exact beginning and end of the pollination of each.

The gravity method has been criticized as giving no real measure of the number, or weight, of pollen grains per unit volume of air, since each species of pollen falls at a different rate. Various formulas have been devised to obtain a volumetric value by applying to the pollen counts correction factors based on the size and shape of the grains, as well as on the surface irregularities of those which are echinated, winged, or sculptured. Scheppegrell[36] first made such an attempt based on Stokes' formula. Errors in his application of the physical principles were discovered by Cocke,[37] and mistakes in the calculations of the latter were pointed out by Dahl and Ellis.[38] The last-mentioned and

Durham[39] have also emphasized the equal significance of variations in the weight and specific gravity. There have been some differences of opinion (Cocke,[27, 32] Durham,[39] Hawes, Small, and Miller,[28] and Dahl and Ellis[38]) as to the advantage or disadvantage of employing mathematical correction factors so as to obtain a more accurate index of the number of pollen grains contained in a unit volume of air. Without entering into the merits of the various claims, one may point out that the entire question is more of academic than of practical import. Sufficient difficulties are encountered in the formulas involving size alone, disregarding the fact that pollen grains of the same species vary somewhat in diameter. But in regard to the effect of spiculation (as with ragweed pollen) or other irregularities of contour on the rate of fall, and hence on the volumetric computations, only rough estimates rather than reliable calculations are available. The same statement holds for differences in specific gravity. With any one species, the twenty-four-hour "gravity count" is an adequate guide to daily fluctuations and to comparisons of the pollen density at one locality with that at another. Furthermore, there is so much variation in the antigenicity of different pollens and in the respective tolerance of individual patients that a comparison of the relative numbers of the various species, even if perfectly accurate, would have no clinical significance. Moreover, it has been amply demonstrated that the inequalities in the counts at two or more locations within the same city, indeed, on different floors of the same building (Wilmer and Cobe[40]), may be far greater than the effect of such corrections. Vaughan,[41] in discussing the attempts at the standardization of pollen counting, concluded that for practical purposes, including comparison of the concentrations in different years, it is sufficient to record the number of grains per unit area, rather than to estimate mathematically the number per cubic yard per twenty-four hours. Certainly, none of the more recent studies gives any reason to abandon this viewpoint. Finally, while the greatest possible exactness in all aspects of this subject is desirable, simple but reliable data on the duration of pollination would far outweigh all quantitative statistics, whether on a volumetric or a unit area basis.

COMMENT

On the basis of the facts given above, what recommendations can be made concerning the most effective means of increasing our knowledge of phenologic phenomena as they relate to hay fever? It must be apparent that neither field studies nor pollen counts alone are adequate. Both must be simultaneously performed at numerous, carefully chosen stations in the given city or region and the findings correlated. In this way, they supplement each other, so that a rise in the total grass pollen count, for example, can be properly attributed to the particular species observed to be pollinating at that time. Contrariwise, the counts act as a check on mistaken conclusions as to the abundance, profuseness of pollination, or buoyancy of the pollen of various plants, as determined by botanic observations. The identification of both plants and pollens must be accurate, and scientific names employed. The exact technique and the unit of recording of the counts are in themselves of relatively little importance, but adherence to a single unit would obviously be highly advantageous. If correction factors are used, they should be clearly stated, and the basis of the computations

given. There is little to be gained by listing every anemophilous species in a locality, since in the present state of clinical knowledge many of them are of no significance. Too much emphasis should not be placed on daily variations in the count, for, as outlined above, this information is of little practical value. The stress should rather be on the determination of the type of pollen in the vicinity of potential or actual hay fever sufferers, just when each first appears and when each can no longer be found in the air. Since the pollination season varies from year to year,[1] such studies should be continued for a number of successive years so as to accumulate information, first, as to the usual or average dates, and, second, as to the amount of variation which occurs or is likely to occur. Data of this nature are immediately useful in selecting pollens for testing and treatment, in evaluating patients' symptoms, and in planning preseasonal, coseasonal, and perennial therapy.

Rosendahl, Ellis, and Dahl[42] summarize the requisites as follows: "From a clinical standpoint, it is of importance that pollen data demonstrate the following facts: 1. The approximate time (variability is not great) at which a specific kind of pollen annually makes its appearance in the air and when it reaches clinically significant concentration. 2. The duration of the period for which clinically significant amounts are present. 3. The dates (approximate) at which the concentration drops below the level of clinical significance."

While agreeing in principle, we must point out that there is no present method of determining the clinically significant concentration of pollen. While, on a rough statistical basis, the symptoms of a large group of sufferers parallel the count for their specific pollen, the correlation is a crude one, and a considerable percentage of patients seem to run contrary, or at least independently during the season, particularly in its latter portion. There is every reason to believe that the pollen incidence capable of eliciting symptoms will vary greatly for different patients: for the same patient from time to time; for different species; and will change according to the state of the offending pollen grains (dry or moist, fresh or old).

But even the most accurate, most reliable pollen data would still leave much to be desired in our evaluation of the importance of each species in pollinosis. It is a well-known fact that a pollen may be abundant and buoyant, as for example that of pine, and still give rise to little or no hay fever. This, of course, corresponds to Thommen's[43] first postulate. Only by the simultaneous collection of pollination data and the clinical investigation of large groups of patients in the same area will any basis be reached for judgment. For this purpose, statistical studies of skin reactions suggest themselves as the most readily applicable and simplest method. But skin tests alone are inadequate, since cutaneous and clinical hypersensitiveness need not always coincide. It is now recognized that a certain proportion of pollinosis cases give positive cutaneous responses to species which elicit no other allergic manifestations in them, and even to those with which, for geographic reasons, they could never have come into contact. Skin reactions should therefore be compared with the presence or absence of symptoms during the pollinating period of the given plant. In addition, nasal and conjunctival tests may often be profitably resorted so as to confirm the reaction with that of other tissues. It is likewise well-known that

some cases will fail to react to the pollens to which they are hypersensitive. Once the diagnosis of hay fever is established in such cases, the determination of the etiologic agent will depend on careful evaluation of the entire clinical picture, with special emphasis on the patients' "seasons," fluctuations in symptomatology as related to variations in pollen pollution, the response to specific therapy, and the inadvertent elicitation of constitutional reactions with specific extract. The possible difficulties encountered in evaluating the results of tests and in establishing the etiologic diagnosis are discussed by the authors[44] elsewhere. Despite these problems, it is only by a correlation of concurrent phenologic and clinical data that any safe conclusions can be reached as to the relative importance in pollinosis of each species of tree, grass, or weed.

Where may such pollen studies most profitably be performed? If the intention is merely to fill in the existing gaps, a glance at the maps and lists included will provide the answer; however, a coordinated nationwide survey combining botanic observations, pollen counts, and clinical investigations, according to a uniform technique, would yield a mass of valuable information and go far to resolve the present confusions and contradictions in this subject. The equipment required and the cost of such a study would be negligible in comparison to the utilizable knowledge it would provide. It could most advantageously be carried out in the larger centers of population, since there the great majority of hay fever victims reside, and since only there would the necessary facilities be found, along with a sufficient number of patients, to make the clinical data significant. As mentioned, the investigation would have to be continued for a number of successive years.

Very recently the Society for the Study of Asthma and Allied Conditions[45] published a preliminary record of the pollen surveys by their members. This is, indeed, the first collective effort to solve the problem under consideration, and will, when all data are published, undoubtedly prove a major contribution to the aim outlined above.

While the actual work in a study such as the one suggested could be done by allergists and botanists in the various communities, obviously a nationwide organization would be necessary to lay down standards, to decide on a uniform technique, and above all, to analyze and correlate the accumulated data so as to reach clinically useful conclusions. The logical choice for this purpose would be one of the national allergy societies or the Public Health Service.

SUMMARY

A review of the existing fund of information regarding the distribution and pollination times of the pollinosis-producing plants in the United States reveals: (1) many states and large cities where no data or only inadequate data are available; (2) certain unavoidable sources of error in the methods of investigation; (3) common correctable deficiencies in the published reports which greatly diminish their value; (4) an unwarranted emphasis, in some instances, on large numbers of species, rather than on those of clinical importance; and (5) an emphasis on quantitative considerations unjustified by present clinical knowledge or applicability.

A correlated pollen survey based on simultaneous botanical observations, pollen counts, and clinical investigations is suggested. This should be done on a nationwide scale and according to a uniform technique.

REFERENCES

1. Gottlieb, P. M., and Urbach, E.: The Distribution and Pollination Times of the Important Hay Fever-Producing Plants in the United States, J. Lab. & Clin. Med. 28: 1053, 1943.
2. Rowe, A. H.: Botanical Survey of San Francisco, California and West. Med. 30: 173, 1929.
3. Deamer, W. C., and McMinn, H. E.: Studies in Atmospheric Pollen of San Francisco, J. Allergy 6: 480, 1935.
4. Vaughan, W. T., Graham, W. R., and Crockett, R. W.: Hay Fever Prevalencies in Virginia: Review of a Six Year Survey, Virginia M. Monthly 60: 158, 1933.
5a. Blanton, W. B.: Hay Fever With Comments on the Past Season in Richmond, Ibid. 59: 140, 1932.
b. Vaughan, W. T.: Allergy and Applied Immunology, St. Louis, 1931, C. V. Mosby Co., p. 82.
6. French, S. W.: Pollens Which Cause Hay Fever in South Central Texas, J. Allergy 1: 286, 1930.
7. Kahn, I. S.: The Botany of Southwest Texas With Reference to Hay-Fever and Asthma, J. A. M. A. 82: 871, 1924.
8. Koessler, K. K., and Durham, O. C.: A System for an Intensive Pollen Survey With a Report of Results in Chicago, Ibid. 86: 1204, 1926.
9. Rowe, A. H.: A Study of the Atmospheric Pollen and Botanic Flora of the East Shore of San Francisco Bay, J. Lab. & Clin. Med. 13: 416, 1928.
10. Lamson, R. W., and Watry, A.: The Importance of the Chenopodiaceae in Pollinosis With Special Reference to Winslow and Holbrook, Arizona, J. Allergy 4: 255, 1933.
11. Idem: Survey of a Botanical Oasis in the Desert of Northern Arizona, Ibid. 5: 389, 1934.
12. Sellers, E. D.: Pollinosis in the Southwest, South. M. J. 28: 710, 1935.
13. Mullin, W. V.: Pollen and Hay Fever—A Regional Problem, Tr. Am. Acad. Ophth. 27: 467, 1922.
14. Scheppegrell, W.: The Classification of Hay-Fever Pollens From a Biological Standpoint, Boston M. & S. J. 177: 42, 1917.
15. Moore, G. T., and La Garde, R. V. L.: The Identification of Pollens From So-Called "Hay Fever" Plants, Ann. Missouri Bot. Gard. 13: 355, 1926.
16. Piness, G., and McMinn, H. E.: The Rôle of the Structural Features of Pollen Grains in Identifying the Most Important Hay Fever Plants of California, J. Lab. & Clin. Med. 12: 1164, 1927.
17. Hoch, J. H., and Waring, J. I.: "Hay Fever" Plants of Charleston, S. C., and Vicinity, J. South Carolina M. A. 29: 69, 1933.
18. Wodehouse, R. P.: Pollen Grains, New York, 1935, McGraw-Hill Book Co.
19. Idem: The Hay Fever Plants of the South, South. M. J. 34: 986, 1941.
20. Thomas, J. W.: A Comparison of Cedar Oil and Other Materials in the Making of Slides of Atmospheric Pollen, J. Lab. & Clin. Med. 25: 1086, 1940.
21. Alford, R. I.: Pollen Studies: A Contrast Color Method of Examining Unstained Pollen Grains, J. Allergy 12: 572, 1941.
22. Castex, M. R., and Moreno, G. R.: Extensiómetro para portaobjetos, Prensa. méd. argent. 28: 1235, 1941.
23. Oliveiro Lima, A., and Greco, J. B.: A Slide for Counting Atmospheric Pollen, J. Allergy 13: 317, 1942.
24. Duke, W. W.: A Rapid and More Accurate Method of Determining the Pollen Content of the Air, J. A. M. A. 99: 1686, 1932.
25. Durham, O. C.: A Simple Apparatus for Determining the Pollen Content of the Air, The Aerologist 11: 10, 1935.
26. Zifferblatt, A. H., and Seelaus, H. K.: A Study of Air Contaminants in the Interest of Public Health: An Improved Method of Pollen Survey, Pennsylvania M. J. 39: 67, 1935.
27. Cocke, E. C.: A Method for Determining Pollen Concentration of the Air, J. Allergy 9: 458, 1938.
28. Hawes, R. C., Small, W. S., and Miller, H.: An Apparatus for Determining the Pollen Content of Air and Notes on Pollen Survey Methods, Ibid. 13: 474, 1942.
29. Scheppegrell, W.: Airplane Tests of Hay Fever Pollen Density in the Upper Air, M. J. & Record 119: 185, 1924.
30. Idem: Hay Fever Pollens in the Upper Air, Ibid. 121: 660, 1925.
31. MacQuiddy, E. L.: Air Studies at Higher Altitudes, J. Allergy 6: 123, 1935.

32. Durham, O. C.: Methods in Aerobiology, J. Aviation Med. 12: 153, 1941.
33. Acquarone, P., and Gay, L. N.: A Survey of the Pollen Flora in Baltimore During 1929, J. Allergy 2: 336, 1931.
34. Patterson, P. M., and Gay, L. N.: The Pollen Content of the Air and Its Relation to Hay Fever in Baltimore, Maryland, During 1930, Ibid. 3: 282, 1932.
35. Durham, O. C.: The Ragweed Season of 1931: Third Annual Atmospheric Survey, *Ibid.* 3: 357, 1932.
36. Scheppegrell, W.: Hayfever and Hayfever Pollens, Arch. Int. Med. 19: 959, 1917.
37. Cocke, E. C.: Calculating Pollen Concentration of the Air, J. Allergy 8: 601, 1937.
38. Dahl, A. O., and Ellis, R. V.: The Pollen Concentration of the Atmosphere, Pub. Health Rep. 57: 369, 1942.
39. Durham, O. C.: A Critical Interpretation of Data on the Incidence of Air-Borne Allergens, J. Allergy 13: 31, 1941.
40. Wilmer, H. B., and Cobe, H. M.: A Note on the Quantitative Survey of Pollen, Ibid. 3: 63, 1932.
41. Vaughan, W. T.: Practice of Allergy, St. Louis, 1939, C. V. Mosby Co., p. 469.
42. Rosendahl, C. O., Ellis, R. V., and Dahl, A. O.: Air-Borne Pollens in the Twin Cities Area With Reference to Hay Fever, Minnesota Med. 23: 619, 1940.
43. Thommen, A. A.: Part 3 of Asthma and Hay Fever in Theory and Practice, by Coca, A. F., Walzer, M., and Thommen, A. A., Springfield, Ill., 1931, Charles C Thomas.
44. Urbach, E., with the collaboration of Gottlieb, P. M.: Allergy, New York, 1943, Grune & Stratton.
45. Report of the Committee on a Pollen Survey of the United States, The Society for the Study of Asthma and Allied Conditions, J. Allergy 13: 516, 1942.

RELATION OF LENGTH OF CARBON CHAIN TO THE PRIMARY AND FUNCTIONAL TOXICITIES OF ALCOHOLS*

HENRY WELCH, PH.D., AND GLENN G. SLOCUM, PH.D., WASHINGTON, D. C.

IN A previous publication[1] a method was described for determining the toxicity of antiseptics on human or guinea pig blood as a tissue by measuring inhibition of phagocytic activity. Later work[2] showed that in many instances the phagocytic inhibition occurred through destruction of some component or components of the humoral system, while in others the leucocyte itself was destroyed. Following a study[3] of the use of other components of the blood as a measure of toxicity, a new test was described[4] in which toxicity was measured by actual death of the cell (leucocyte). Both of these tests were developed primarily to determine the direct toxic effect, mainly of antiseptics, on whole blood as a tissue. Such tests may be used to determine the "primary toxicity" of a single compound or the "relative primary toxicities" of a group of compounds.

The term toxicity has come to have a broad meaning. It is used to describe not only primary "irritating," "damaging," or "destructive" action on an isolated tissue, as used for instance by Salle et al.[5] in his toxicity test on chick embryo tissue or by Bronfenbrenner et al.[6] with his toxicity method using mouse liver cells, but also is used to designate what might be called the "functional" toxicity of a chemical injected into or fed animals in the assay of such compounds. *Hence Macht[7] describes the "toxicity" of the primary alcohols* for cats when injected intravenously, while Woodard and his collaborators[8] report on the "acute oral toxicity" of acetic and the chloracetic acids. *In contrast to the "primary toxicity" of a compound on tissue cells in vitro which measures*

*From the U. S. Food and Drug Administration, Washington, D. C.
Received for publication, January 7, 1943.

a single isolated toxic action, measurement of "functional" toxicity not only encompasses the sum of the primary toxic effects, but also includes other factors such as the selective affinity of some chemicals for certain organs, cells, or cellular mechanisms of the animal body and the physiologic effects of end products of metabolism of the chemical. Thus where "primary" toxicity is tested on human blood in vitro, using inhibition of phagocytic activity as an index of toxic effect, phagocytosis ceases in the presence of 5 per cent of methyl alcohol. Yet when methyl alcohol is taken into the digestive tract, products probably produced by intermediate oxidation bring about a deleterious effect in the animal which bear no relation to the "primary" toxic effect measured on individual cells. Similarly potassium cyanide which produces death when injected or fed in small amounts has very little effect on the isolated white cell even in high concentrations.

In the present study the primary toxicities of the monohydroxy aliphatic alcohols and the toxicities of acetic, mono-, di-, and trichloracetic acids have been determined by the method previously described,[1] using inhibition of phagocytosis in whole human and rat blood in vitro as an index of toxic effect. These toxicities have been compared with the acute oral toxicities obtained on the acids by Woodard, Lange, Nelson, and Calvery[8] and on the alcohols by Woodard.[*] The rats used were predominantly males and were raised by the Division of Pharmacology of the Food and Drug Administration. They ranged in weight from 150 to 250 Gm. and in age from 70 to 100 days. The alcohols were administered to the rats orally after an eighteen-hour fasting period by means of a soft rubber catheter attached to a syringe. Following treatment the rats were observed for six days, and those surviving were not used in subsequent experiments.

The results obtained in testing the resistance of both human and rat leucocytes to the toxic action of seven normal alcohols and the acute oral toxicity for rats of these same alcohols are given in Table I. It will be noted that as the molecular weight of the alcohols increases, the concentration of the alcohol necessary to bring about complete inhibition of phagocytosis decreases. Whereas methyl alcohol inhibited phagocytosis in a dilution of 1:20, ethyl alcohol prevented phagocytosis in a dilution of 1:30, propyl at 1:70, butyl at 1:175, amyl at 1:450, hexyl at 1:1000, and heptyl at 1:2500. The human and rat leucocyte both showed approximately the same resistance to the toxic action of the alcohols throughout the series. It will also be noted (Table I) that the molarity of the toxic dilution decreases from M/.64 for methyl alcohol to M/290.7 for heptyl alcohol in a fairly regular sequence. When the toxic molar concentration of ethyl alcohol is taken as unity and the relationship of the other six alcohols computed, it will be noted that the toxicity in this homologous series of alcohols for the leucocytes of humans or rats increases in the order of molecular weights following semiquantitatively the geometric progression described by Kamm[9] for these alcohols, $1:3^1:3^2:3^3:3^4:3^5$.

This generalization, i.e., that toxicity in a homologous series increases with the molecular weight, known as Richardson's Rule, has been verified by Morgan and Cooper[10] and by Tilley and Schaffer[11] in testing the germicidal action of

*The authors are indebted to Geoffrey Woodard for the unpublished data on the acute oral toxicities of the primary alcohols in rats.

the aliphatic alcohols, by Macht (loc. cit.) in a study of the acute toxicity of these alcohols when injected intravenously in cats and by Kamm (loc. cit.) who tested their toxicity for paramecia as well as their albumen-coagulating power. In contrast to these results when acute oral toxicity is determined in rats (Table I), there is no regular relationship between toxicity and molecular weight. The alcohols increase in functional toxicity irregularly from methyl through butyl alcohol and then decrease in toxicity from amyl to heptyl alcohol. These results emphasize the essential difference between "primary" and "functional" toxicity. In the case of the latter, the fate of the animal is determined by a variety of factors including the solubility, rate of absorption and excretion, and the effect of body fluids on the alcohol.

TABLE I

TOXICITY OF PRIMARY ALCOHOLS

ALCOHOL	TOXICITY AS MEASURED BY INHIBITION OF PHAGOCYTOSIS IN WHOLE BLOOD*			ACUTE ORAL TOXICITY IN RATS†
	TOXIC DILUTION	MOLARITY OF TOXIC DILUTION	RATIO MOLARITY TOX. DIL. TO MOLARITY TOX. DIL. OF ETHYL ALCOHOL	GM. PER KG. OF BODY WEIGHT
Methyl	1:20	M/.61	.46	9.1
Ethyl	1:30	M/1.38	1.00	7.4
Propyl	1:70	M/4.21	3.05	3.3
Butyl	1:175	M/12.96	9.40	2.75
Amyl	1:450	M/39.82	28.85	3.3
Hexyl	1:1000	M/102.01	73.98	4.1
Heptyl	1:2500	M/290.7	210.76	Approx. 6.6

*Both human and rat blood were used in these tests. Equal resistance of leucocytes to the toxic effect of these alcohols was demonstrated.

†Acute oral toxicity was determined by Geoffrey Woodard, Division of Pharmacology, U. S. Food and Drug Administration.

In order to demonstrate further the difference between what has been designated in this report as "primary" toxicity for an isolated tissue or cell and "functional" toxicity, a study has been made of acetic acid and the chloracetic acids in the same manner as the alcohols were investigated. The ability of acetic acid and the chloracetic acids to inhibit phagocytosis completely in whole human and rat blood has been determined in both acid and neutral solutions, and these results compared with the acute oral toxicity in rats as determined by Woodard et al. (loc. cit.). The results are shown in Table II, in which it will be noted that acetic acid is the most toxic and monochloracetic, dichloracetic and trichloracetic acids decrease in toxicity in that order for both human and rat leucocytes. The rat leucocyte is more susceptible to the action of these chemicals than the human. The pH of the toxic dilution decreases from 3.18 for acetic acid to 1.73 for trichloracetic acid with human cells and from 3.23 to 1.85 with rat cells indicating that the toxicity is probably related to the undissociated molecule rather than to the acidity. Computation of the molarity of the toxic dilution of all four acids, i.e., that dilution causing complete inhibition of phagocytosis, indicates that within experimental error all of these acids are toxic in equimolecular concentrations.

Since it was advisable to determine the acute oral toxicities of the acid radicals and not their toxicities as acids, the acids were neutralized with sodium

hydroxide to a pH range between 6 and 7. Neutral solutions of this type were also tested for their primary toxicity for both human and rat leucocytes. These results are also given in Table II where it will be noted that the same dilution (1:70) of all four neutralized acids inhibited phagocytosis of the human leucocyte, while a 1:100 dilution of each acid prevented phagocytosis by the rat leucocyte. In direct contrast to the equivalent primary toxicities of these four neutral acids for the rat white cell, are the results obtained on feeding these acids to rats, since it will be noted that monochloracetic acid is by far the most toxic, dichloracetic the least toxic, while acetic and trichloracetic have approximately equal "functional" toxicities.

TABLE II

TOXICITY OF ACETIC AND CHLORACETIC ACIDS

ACID	HUMAN BLOOD				RAT BLOOD				RATS
	ACID SOLUTIONS			NEUTRAL SOLUTIONS	ACID SOLUTIONS			NEUTRAL SOLUTIONS	NEUTRAL SOLUTIONS
	TOXIC DILUTION	PH TOXIC DILUTION	MOLARITY TOXIC DILUTION	TOXIC DILUTION	TOXIC DILUTION	PH TOXIC DILUTION	MOLARITY TOXIC DILUTION	TOXIC DILUTION	*ACUTE ORAL TOXICITY IN GM./KG. OF BODY WEIGHT
Acetic	1:550	3.18	.03	1:70	1:700	3.23	.024	1:100	3.31
Monochloracetic	1:400	2.27	.026	1:70	1:650	2.46	.016	1:100	.0762
Dichloracetic	1:300	1.77	.026	1:70	1:400	2.00	.019	1:100	4.48
Trichloracetic	1:250	1.73	.025	1:70	1:300	1.85	.020	1:100	3.32

*Values obtained by the Division of Pharmacology, U. S. Food and Drug Administration. J. Indust. Hyg. & Toxicol. 23: No. 2, 1941.

COMMENT

The method described previously[1] for measuring the toxicity of antiseptics or other chemicals brought into direct contact with tissue, i.e., by determining that concentration of the compound under test which completely inhibits phagocytosis, may be considered to be an estimation of "primary" toxicity on an isolated cell. This toxic action may manifest itself by a destructive effect on some component or components of the humoral system or by actual destruction of the cell itself.[4] In either case the toxic action measured is on an isolated individual tissue cell. When the primary aliphatic alcohols are tested by this technique, a definite and uniform increase in toxicity is observed with increase in molecular weight. These results are in accord with those of Kamm (loc. cit.), who found the toxicity of the alcohols for paramecia to be in proportion to their molecular weight, while similar results were obtained by Tilley and Schaffer (loc. cit.) in determining the destructive action of these alcohols for *Eberthella typhosa* and *Staphylococcus aureus*. From these studies with leucocytes, protozoa, and bacteria, it is evident that when the normal alcohols are tested for their primary toxicity for individual cells, a definite relationship between molecular weight and toxic activity can be readily demonstrated.

Similarly Macht (loc. cit.) demonstrated an increase in acute toxicity with increase in molecular weight when these alcohols were injected intravenously into cats, although the increase in toxicity with increased molecular weight does

not appear to follow a definite mathematical pattern. Thus Macht reports n-propyl alcohol to have a toxicity of 2.5 times and n-butyl 16.6 times the toxicity of ethyl alcohol. Kamm (loc. cit.) points out that the abnormalities in Macht's results for n-butyl alcohol, i.e., the fact it does not follow the geometric progression $1:3^1:3^2:3^3$, may be ascribed to a too concentrated solution of this material when injected intravenously. It may be, however, that the abnormalities noted with butyl alcohol injected intravenously into cats are associated with the method of test, and the discrepancies, an indication of the selective action of this alcohol for some vital organ. When acute oral toxicity is measured in rats, an entirely different result is obtained. The toxicities of the first four normal alcohols follow Richardson's rule, while the last three tested failed to do so. In fact, amyl, hexyl, and heptyl alcohols decreased in toxicity in that order. Undoubtedly the lower solubilities of the higher alcohols have a marked effect on oral toxicity.

When a comparison is made of the primary toxicity, i.e., toxicity for rat tissue and the acute oral toxicity of the neutralized acetic acid and chloracetic acids for rats, we again note a marked difference in the results obtained. While monochloracetic acid has an acute oral toxicity approximately 40 times that of the other three acids, the primary toxicity for rat leucocytes was found to be the same for all four acids. These results indicate that there is a marked difference between "primary" toxicity as measured by the action of a compound on an isolated tissue cell and "functional" toxicity in which the compound is fed or injected into an animal and hence influenced either by a selective affinity of the compound for some vital organ of the body or by the physiologic action of body fluids.

SUMMARY AND CONCLUSIONS

1. The primary toxicities of the monohydroxy aliphatic alcohols, acetic acid and chloracetic acids against human and rat blood as tissues have been contrasted with the acute oral toxicities of these chemicals for rats.

2. Human and rat leucocytes are equally susceptible to the primary toxic action of the alcohols, and the toxicity of the alcohols for these cells increases with molecular weight. This increase of toxicity with increase in molecular weight follows closely the geometric progression $1:3^1:3^2:3^3:3^4:3^5$ noted by Kamm.

3. The acute oral toxicity to rats of the first four primary alcohols increases with increase in molecular weight, while amyl, hexyl, and heptyl alcohols decrease in toxicity with increase in molecular weight.

4. Rat leucocytes are more susceptible than human leucocytes to the primary toxic action of acid and neutral solutions of acetic acid and the chloracetic acids. Acid solutions of the four acids were toxic for leucocytes in approximately equimolecular concentrations. Neutral solutions of the four acids were toxic for leucocytes in the same concentrations.

5. The acute oral toxicity to rats of acetic acid and the chloracetic acids bears no relation to molecular weight, since monochloracetic acid is approximately forty times more toxic than the other members of the series.

6. There is a marked difference between "primary" toxicity as measured by the direct action of a chemical on an isolated tissue cell and "functional"

toxicity as determined by the administration of the chemical where the toxic action is directed against the body as a whole.

REFERENCES

1. Welch, Henry, and Hunter, A. C.: Method for Determining the Effect of Chemical Antisepsis on Phagocytosis, Am. J. Pub. Health 30: 129, 1940.
2. Welch, Henry: Mechanism of the Toxic Action of Germicides on Whole Blood Measured by the Loss of Phagocytic Activity of Leucocytes, J. Immunol. 37: 525, 1939.
3. Welch, Henry, Brewer, C. M., and Hunter, A. C.: Toxicity of Antiseptics; Experiments With Hemolytic Complement, J. Immunol. 38: 273, 1940.
4. Welch, Henry, Slocum, G. G., and Hunter, A. C.: Method for Determining the Toxicity of Antiseptics as Measured by the Destruction of Human Leucocytes, J. Lab. & Clin. Med. 27: 1432, 1942.
5. Salle, A. J., McOmie, W. A., and Shechmeister, I. L.: A New Method for the Evaluation of Germicidal Substance, J. Bact. 34: 267, 1937.
6. Bronfenbrenner, J., Hershey, A. D., and Doubly, J. A.: Evaluation of Germicides by a Manometric Method, Proc. Soc. Exper. Biol. & Med. 38: 210, 1938.
7. Macht, David I.: A Toxicological Study of Some Alcohols, With Especial Reference to Isomers, J. Pharm. & Exper. Therap. 16: 1, 1920.
8. Woodard, Geoffrey, Lange, S. W., Nelson, K. W., and Calvery, H. O.: The Acute Oral Toxicity of Acetic, Chloracetic, Dichloracetic and Trichloracetic Acids, J. Indust. Hyg. & Toxicol. 23: 78, 1941.
9. Kamm, Oliver: The Relation Between Structure and Physiological Action of the Alcohols, J. Am. Pharm. 10: 87, 1921.
10. Morgan, G. J., and Cooper, E. A.: The Influence of the Chemical Constitution of Certain Organic Hydroxyl and Aminic Derivatives on Their Germicidal Power, Eighth Int. Cong. Appl. Chem. 19: 243, 1912.
11. Tilley, F. W., and Schaffer, J. M.: Relation Between the Chemical Constitution and Germicidal Activity of the Monohydric Alcohols and Phenols, J. Bact. 12: 303, 1926.

AN UNUSUAL TEMPERATURE COURSE IN INFECTIOUS MONONUCLEOSIS*

SEYMOUR H. RINZLER, M.D., AND JULIUS J. HERTZ, M.D., NEW YORK, N. Y.

SIR WILLIAM OSLER dubbed syphilis the great mimicker of other diseases. Infectious mononucleosis with its protean manifestations is fast becoming a successful runner-up to lues in this regard. Bernstein,[1] in his excellent review of infectious mononucleosis, demonstrates that this disease may affect almost all of the body systems. Because the recording of each new manifestation of this disease will lead to its complete understanding, the report of an unusual temperature curve is presented.

REPORT OF CASE

A white male aged 25 years was first seen on the sixth day of his illness. He complained of malaise, fever, and swelling of the glands of the left side of the neck. Physical examination revealed a temperature of 101° F. and enlarged glands of the left posterior cervical chain. Excepting for later involvement of the right posterior cervical and right occipital lymph glands, the physical examination was entirely negative throughout the course of the illness. On the twelfth, fourteenth, sixteenth, eighteenth, and twenty-second evenings of the illness (Fig. 1), the patient had a sudden chill, violent enough to shake the entire bed, followed by a rapid rise in the temperature to levels between 101° F. to 104.8° F. and profuse sweating. In each instance the temperature fell by crisis within a period of two

*Received for publication, January 18, 1943.

to three hours. The chill, which lasted from ten to twenty minutes, was always preceded by an ache in the lower back which increased in intensity as the temperature rose and subsided as the temperature fell. The white blood count on the eleventh day of the illness was 7,950 with 53 per cent neutrophils (38 per cent segmented; 15 per cent nonsegmented), 40 per cent lymphocytes, and 7 per cent monocytes. The heterophile antibody reaction was negative. Two smears for malarial parasites were negative. On the thirteenth day a blood culture was negative, and agglutination reactions for typhoid "O," typhoid "H," paratyphoid "A," paratyphoid "B," proteus X19 (typhus fever), and *Brucella abortus* were negative in all dilutions. On the fifteenth day of the illness, the heterophile antibody reaction was positive in a dilution of 1:64. The white blood count was 14,700 with 28 per cent neutrophils (24 per cent segmented; 4 per cent nonsegmented), 1 per cent eosinophiles, 51 per cent small lymphocytes, 18 per cent pathologic lymphocytes, and 2 per cent monocytes. The presence of these pathologic lymphocytes, which were described by Downey and McKinlay,[2] is characteristic of infectious mononucleosis. Several urinalyses were negative. After the chill and fever of the twenty-second day, the temperature fell to normal and remained there. The patient made an uneventful recovery.

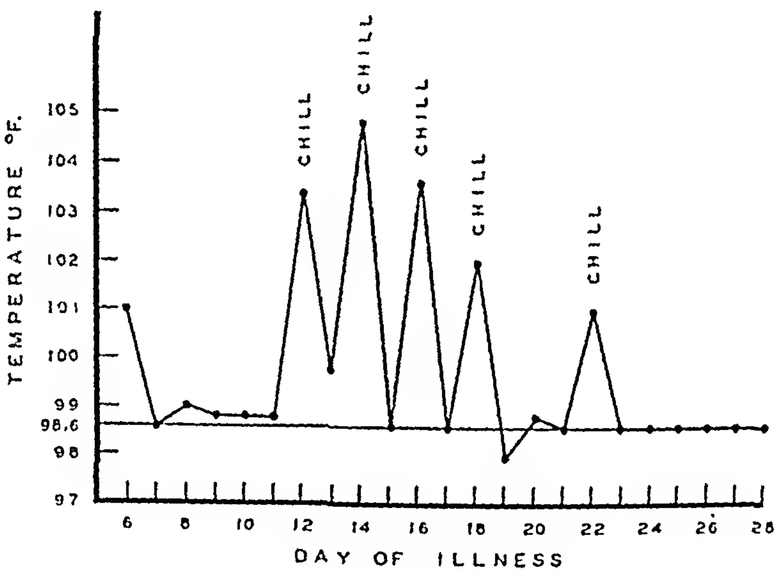


Fig. 1.—Each point represents the highest temperature for that particular day.

COMMENT

Bernstein,[1] in whose monograph on infectious mononucleosis the literature to 1940 is reviewed, does not mention such a temperature course. He further states[3] that in his personal experience he has never seen this disease behave in this way. Anyone who has dealt with the problems in the differential diagnosis of this disease may readily understand our difficulties until the true nature of the illness revealed itself. Our first clinical impressions included infectious mononucleosis, but the heterophile antibody reaction was still negative on the eleventh day. Then the series of chills and fever began. The possibility of malarial infestation was ruled out by two negative blood smears for the presence of the parasites. Sepsis and the enteric fevers were eliminated by the negative blood culture and agglutination tests. The lymphadenopathy and lymphocytosis brought to mind the possibilities of either an acute lymphatic leucemia or an acute Hodgkin's disease. The diagnosis was finally established by the positive

heterophile antibody reaction in a dilution of 1:64 and the presence of the abnormal lymphocytes[2, 4] in the blood smear. Whether or not suppuration or the breaking down of the retroperitoneal lymph glands was the cause of both the temperature response and the backache could only be surmised.

SUMMARY

A case of infectious mononucleosis with an unusual temperature course resembling that of malaria is presented. The absence of any previous mention in the literature of such a temperature response is noted.

REFERENCES

1. Bernstein, Alan: Infectious Mononucleosis, Medicine 19: 85, 1940.
2. Downey, Hal, and McKinlay, C. A.: Acute Lymphadenosis Compared With Acute Lymphatic Leukemia, Arch. Int. Med. 32: 82, 1923.
3. Bernstein, Alan: Personal communication to the authors.
4. Downey, Hal: Infectious Mononucleosis, Symposium of the Blood and Blood Forming Organs, University of Wisconsin Press, 1939, p. 194.

ATTEMPTS TO OBTAIN BETTER RESULTS WITH THE BACTERIAL ANTIGEN ("VACCINE") THERAPY OF LOW GRADE CHRONIC ("FOCAL") INFECTION

I. POSSIBLE ERRORS OF USUAL METHODS

MERRITT H. STILES, M.D., F.A.C.P.* CONRAD BERENS, M.D., F.A.C.S.,† WILLIAM B. RAWLS, M.D., F.A.C.P.,‡ and GEORGE H. CHAPMAN,§
NEW YORK, N. Y.

DURING the past seventeen years we and our associates have been making a careful study of the problem of low grade chronic ("focal") infection in an effort to determine its significance. Such a study included investigation of the field of bacterial antigen ("vaccine") therapy and has resulted in the development of diagnostic and therapeutic bacteriologic methods which we believe are superior to others we have tried. This series of reports presents a discussion of the progress achieved so far.

Although most physicians believe that there is little merit in the "vaccine" therapy of low grade chronic infections, its continued use by some suggests that it must have value. Differences of opinion as to its value might be based on differences in the technique of preparing and administering the "vaccines." That there may be room for improvement in manufacture, even in standardized vaccines that have been used extensively for a long time, is indicated by the re-

*Major, Medical Corps, U. S. Army.

With the cooperation and advice of Drs. Earl H. Eaton, Anthony A. Ressa, H. F. Rubenstein, and Edward C. Titus.

Aided by grants from the Ophthalmological Foundation, Inc.

†Professor, Department of Ophthalmology, New York University Medical School. Ophthalmic Surgeon and Pathologist, New York Eye and Ear Infirmary.

‡Chief, Arthritis Clinic, New York Polyclinic Medical School and Hospital. Chief, Arthritis Clinic, City Hospital, New York, N. Y.

§Director, Clinical Research Laboratory, New York, N. Y.

Received for publication, January 26, 1943.

cent radical change in the type of organism used in the preparation of typhoid vaccine.[1] Our findings indicate that the failure to secure results in bacterial antigen therapy of chronic low grade infections may be caused by: selecting cultures that are incapable of inciting the production of suitable antibodies or of suitable quantities of them; improperly treating the cultures; failing to provide adequate surgical and supportive measures; and using unsatisfactory dosage with respect to both the interval and dose. More specifically, the following factors may have a profound effect on the course of such treatment.

POSSIBLE BACTERIOLOGIC ERRORS

a. *Culture of unsuitable foci.*—In the search for possible foci of infection a significant focus may be overlooked and cultures may be made of other foci. For example, in a patient with chronic prostatitis and rheumatism the prostate was considered the obvious focus, but only nonpathogenic organisms were found in the prostatic fluid. Highly pathogenic organisms were found in the nasal and oral cavities, which were the seat of long-standing but almost asymptomatic infection. Quite often special attention is paid to one particular focus, while others may be ignored.

If, as seems reasonable on the basis of available data,[2] "*Streptococcus viridans*" is the common denominator of low grade chronic infections, then knowledge of the incidence of pathogenic types of this organism should prove useful in indicating their most likely sources. Ruggier and Chapman[3] found resistant (i.e., probably pathogenic) streptococci to comprise 72 per cent of streptococci from periapical pockets, 54 per cent of those from extracted teeth, 44 per cent of those from the pharynx, 35 per cent of those from the nasopharynx, and 28 per cent of those found in the feces ("enterococci" being ignored). By use of an improved technique pathogenic types of streptococci were found in the pharynx in *every* person with low grade infection. For this reason the pharynx (and any periapical pockets or extracted teeth), should be cultured in every case. (The most common source of pathogenic staphylococci is the nasal cavity, which likewise should be cultured routinely.) Additional cultures should be taken from other sources whenever there is suspicion of active infection. For example, in one patient there were only 200,000 pathogenic type streptococci (18 per cent of the total streptococci in the swab) in a swab from the pharynx, but there were 5,000,000 (36 per cent of those in the gingival swab) in a swab from the gum margins, many of which showed evidence of inflammation. (See also Ruggier and Chapman.[3])

b. *Use of improper cultural methods.*—Laboratories that are infrequently called upon to make cultural studies of focal infection have difficulty in maintaining a supply of suitable culture media. Hence some of them use the Loeffler medium slants, supplied by departments of health, which are frequently dry and unsatisfactory for this type of work. Others use "blood agar" plates of varying composition, poured so thin, or which are so old, that they are dry and unsuitable for adequate growth of pathogenic bacteria, many of which require abundant moisture. Usually the material is spread on only one plate, resulting in a crowded growth that makes the study of individual colonies difficult.

Unless appropriate cultural methods are used, streptococci, particularly anaerobic types, pneumococci, some members of the Hemophilus group, and other

fastidious organisms may not be recovered. Much tedious work can be eliminated by using the newer selective isolation methods. Penicillin, recently discussed by Chain et al.,[4] and Abraham et al.,[5] is useful in the selective isolation of the Hemophilus group. Bromthymol blue lactose agar and phenol red mannitol agar[6, 7] give superior results in the selective isolation of probable pathogenic staphylococci. Crystal violet, sodium azide, tryptose blood agar[8, 23] is of considerable assistance in isolating pathogenic streptococci from mixed cultures.

c. *Taxonomic errors and unsuitable taxonomic considerations.*—Often an organism diagnosed as "hemolytic streptococcus" may be a hemolytic member of the Hemophilus group, a hemolytic staphylococcus, or even an α- or γ-hemolytic streptococcus. The presence of a small hemolytic colony on the *surface* of "blood agar" does not warrant a diagnosis of "hemolytic streptococcus." Similarly, a mucoid colony of staphylococci or of streptococci is sometimes mistaken for *Klebsiella pneumoniae*. Organisms received as "staphylococci" were found to be "enterococci" and *Neisseria catarrhalis*, and a "Type IV pneumococcus" proved to be *Streptococcus salivarius*.

Sometimes an organism is designated by an insignificant property. For example, numerous publications refer to "hemolytic" staphylococci but it has been shown[9] that the power of staphylococci to produce hemolytic zones on blood agar has only secondary significance.

d. *Error of random selection of colonies.*—When colonies are selected at random from a mixed culture they may not be representative of apparently similar colonies elsewhere on the plate. Only rarely in this type of work do we deal with cultures in which all the cells have similar properties. Therefore, in selecting a colony at random, even from an apparently pure culture, it is possible to select one that does not represent the entire culture.

e. *Influence of the age of the culture.*—Most bacteriologists harvest a culture after "overnight" incubation, an interval which may include extremes of sixteen and thirty hours. Such differences in age are often accompanied by differences in properties of the cultures.[10, 11] (We have found that late logarithmic cultures make more efficient antigens.) Metabolites, particularly hydrogen ions, may have an unfavorable effect on bacteria and their antigens. Polysaccharides, which may be related to the specificity of a bacterial antigen, are not produced uniformly throughout the period of growth of the culture. Autolytic products, the result of prolonged incubation, may alter the antigenic complex. For these reasons, it is important to know the influence of age on the antigenic structure of different cultures.

f. *Fallacy of considering the predominant organism as the focal invader.*—Many bacteriologists in medical fields consider only the gross features of a culture and make no more than a casual inspection of the plates. The relative number of the different types of organisms is estimated on the assumption that the organisms present in largest numbers are most likely to be the focal invaders. While this is often true, the rich growth of saprophytes in many parts of the body precludes the possibility of recognizing focal bacteria on the basis of predominance.

g. *Inadequacy of methods used to differentiate etiologic bacteria from contaminants.*—Because cultures from suspected foci often contain bacteria which

have no relationship to the infection, it is important that attempts should be made to differentiate these contaminants from the organisms causing the infection.

Intradermal tests, complement fixation reactions, agglutination reactions, and qualitative bacteriostatic tests have been shown to be inadequate for such differentiation.[12, 13] There is no evidence that organisms showing specific localization produce better antigens than do those not localizing in particular tissues.

Our researches[17] indicate that differentiation between causative organisms and contaminants can be made by methods based on the probability that bacteria causing infection are more likely than are contaminants to possess properties associated with pathogenicity. (It is possible that strains of low pathogenicity may be responsible for infections in tissues having low resistance; but such instances are uncommon and only a mild infection usually results.) Because of the difficulty of applying animal inoculation tests, studies were made of in vitro properties. It was found that certain of these properties were parallel with pathogenicity for animals,[7, 14, 17] were parallel with other findings[7, 9, 16, 17, 23] in the patients from whom they had been isolated, and, because they were easily reproducible, were suitable for studying large numbers of cultures.

h. *Selection of a nonpathogenic variant.*—Even though accurate methods are used to differentiate pathogenic from nonpathogenic strains, the culture selected may still be nonpathogenic because pathogenic cultures often contain nonpathogenic variants. If different colonies are tested separately, an apparently pure culture will be found in many cases to consist of a mixture of dissociants (or variants) in which different properties may be distributed in different patterns among the different cells of the culture.[3] Because this phenomenon occurs so frequently in α- and γ-hemolytic streptococci, cultures of these organisms should be considered, not as stable homogeneous masses, but as dynamic mixtures of cells with a strong tendency to instability of the biochemical, physiologic, immunologic, and pathogenic properties of the different cells of the cultures. To be fully representative, a culture must not only be genetically related to the etiologic (pathogenic) culture but must also possess its pathogenic properties.

Not much attention appears to have been paid to the possibility that the antigenic structure of a culture may change along with the dissociation of other characters. If the colony or colonies selected for preparing the growth for the antigen should lack any of the antigenic properties of the parent culture, then the antibodies produced from injection of this antigen may be qualitatively and quantitatively different from those necessary to neutralize the antigenic complex produced by the invading microorganisms.

LACK OF SPECIFICITY OF THE ANTIGEN

Even though considerable care is exercised in selecting the culture, there is still a possibility that the antigen prepared from it may not be efficient for the following reasons:

a. *There may be loss or degeneration of the antigenic properties during cultivation of the microorganism.*—Any degeneration of the culture during growth is likely to be associated with degeneration of the antigenic complex. Therefore,

steps should be taken to minimize degeneration by preventing excessive variations in hydrogen-ion concentration and excessive oxygenation, and by harvesting the culture as quickly as possible.

b. *Physical or chemical changes may be produced in the antigen after the culture has been harvested.*—Heat so injures many bacterial antigens that it is necessary to inject several million bacteria to produce a physiologic effect. In our experience 0.5 per cent phenol does not impair the immunogenic properties of staphylococci, streptococci, and Klebsiella, and does not appear to be injurious to the patients in the dosages used. Only rarely do we find sensitivity to phenol. With properly prepared antigens a physiologic effect may be produced with amounts representing less than a single bacterial cell. The antigens should be stored in resistant glass bottles to minimize the accumulation of alkali.

The essential property of a bacterial antigen is that it should be an efficient antibody-stimulating preparation. If the concentration of specific antigen should be too small (as in heated or nonspecific vaccines), the dose required to provoke a high titer of antibodies will contain so much protein that the protein itself may produce local or even general reactions.

UNSUITABLE METHODS OF VACCINE ADMINISTRATION

a. *Fallacy of attempts to force the patient to produce a high titer of antibodies.*—The usual aim in vaccine therapy is to force the patient to produce large amounts of antibodies. For this reason most schedules call for the administration of progressively larger doses to the limit of tolerance, even though the immediate results should be unfavorable. Not only is an unfavorable effect ignored, but quite often it is considered a favorable sign, an indication that the vaccine is having an effect on the patient.

On the contrary, such an unfavorable effect, if more than transitory, usually indicates that the patient is unable to produce a sufficient quantity of antibodies to neutralize the antigen. Consequently the excess antigen acts as a toxin. Recovery might be possible if sufficient opportunity were given for the tissues to produce more antibodies, but often the patient is made worse by another injection before he has had a chance to recover. When an injection is given too soon after the previous one it augments the residual antigen, resulting in even greater toxicity.

b. *Use of empirical methods for determining dosage.*—Because the laws governing response to the introduction of an antigen into the body are poorly understood and because of the difficulty in determining the precise effects, it is customary in administering antigens to follow a fixed schedule such as is suggested by manufacturers of biologic products. While such a procedure works satisfactorily in the prophylactic immunization of healthy persons (e.g., against typhoid), it is not reasonable to expect that it would work equally well in immunizing body tissues against an organism with which they are already infected. The varying amounts of antigen produced by the pathogenic organisms present, the varying damage done to tissues by these organisms, as well as the varying effects of nutritional and endocrine deficiencies, combine to alter the ability of the body to respond to the introduction of additional antigen. In our experience best results have been obtained by adjusting the dosage (i.e., both the dose and

the interval) according to the response to each injection of antigen. This subject, including our methods of estimating response to antigen injections, will be discussed in a later paper.

c. *Improper spacing of injections.*—The response to an injection of antigen is a function of the properties of the antigen, which are constant, and of the tissue reactability and antibody concentration, which vary with the interval. Therefore, it is important to space the injections to take advantage of these variations. If an antigen injection is given too soon after a previous injection, enough unneutralized antigen may still remain in the tissues to make the amount being injected too large. If given too late, the antibody level may have dropped to such a low level that several injections may be required to overcome this slump and obtain optimum response.

d. *Errors in the size of the dose of antigen.*—An inadequate dose of antigen will have only weak power of stimulating the production of antibodies. The closer a dose is to the optimum, the greater will be the degree of stimulation. However, maximum stimulation comes only from a quantity of antigen which is close to an overdose. Consequently, considerable care must be exercised when a particular dose produces marked stimulation, because a slightly higher dose, or the same dose repeated too soon, may cause unfavorable symptoms.

At times, following the administration of a dose which is considerably too large, the signs of overdosage may not be dramatic and may be mistaken for failure to respond to the antigen injection. Even if such a dose is repeated several times, the evidence of overdosage may be difficult to detect. We have seen a number of patients, usually of low vitality, who showed no evident change, either beneficial or otherwise, during a series of antigen injections, but who improved after an interval of several weeks without injections. Later these patients responded satisfactorily to smaller doses of antigen.

During an acute illness, or shortly after surgical removal or treatment of foci of infection, there is often so much circulating toxin that even minute amounts of antigen act as overdoses. Consequently, great care must be taken in giving injections in such cases. In general, it is advisable to postpone antigen therapy until a week after any acute illness has subsided, or until the immediate unfavorable effects of a surgical procedure have disappeared and improvement is evident.

In the early stages of treatment favorable response to the injection of antigen follows a curve: increasing improvement, followed by gradual return to the basic level. An injection of antigen will produce better results if it is given at the end of the period of improvement rather than after complete return to the basic level. When the immunity has been raised to a satisfactory level, the antigen may be given in rapidly increasing doses at frequent intervals.

INADEQUATE SUPPORTIVE MEASURES

a. *Failure to reduce the toxic load.*—Response to the injection of an antigen depends not only on considerations of the antigen but also on tissue reactability, which in turn is affected by the toxic load on the system. An undrained (i.e., confined) focus of infection is a source of toxic material. When the focus is

drained or removed much of this is eliminated, lightening the toxic load on the body. This is one of the most important phases of bacterial antigen therapy. In many instances in which the patient is sensitive to extremely small doses of antigen it will be found that there is an untreated focus. Numerous patients who reacted badly to small doses of antigen had a beneficial response to much larger doses of the same antigen after foci of infection had been drained or removed.

The toxic load may also be reduced by appropriate chemotherapy and physiotherapy.

b. *Failure to correct endocrine dysfunction.*—A lowered basal metabolic rate has been reported as a common finding in chronic arthritis[18] and in chronic low grade infections.[19] In patients who become drowsy after a dose of antigen that is higher than the optimum amount, the drowsiness may often be reduced by giving adequate amounts of thyroid preparations simultaneously with the vaccine injection. In patients with more definite evidence of thyroid hypofunction the continued use of thyroid preparations may prove markedly beneficial. Often, in these cases, thyroid function improves as the general health improves, and thyroid therapy may eventually be discontinued.[20]

Sex gland and adrenal deficiencies may also require attention.

c. *Failure to correct liver dysfunction.*—Liver dysfunction is common in arthritis[21] and other forms of low grade chronic illness.[22] Dietary adjustment (low fat and cholesterol, high carbohydrate and high protein) and the administration of bile salts are useful in correcting liver disturbances and in improving the response to vaccine therapy.

d. *Failure to supply adequate nutrition.*—It is generally recognized that resistance to infection is dependent to a certain extent upon nutritional factors. Dramatic response is sometimes noted when an adequate diet is supplied, or even when a single specific dietary factor is administered. The beneficial effects of the appropriate use of vitamin B complex have been especially noteworthy.

With careful attention to the details enumerated and by application of the principles to be discussed in the following papers, we believe it possible to obtain better results in the bacteriologic investigation and treatment of low grade chronic infections than are possible by other methods that have been proposed for this purpose.

SUMMARY

An analysis of some of the factors that might prevent optimum response to the bacterial antigen ("vaccine") therapy of low grade chronic ("focal") infections reveals the following possibilities:

The cultures may be taken from unsuitable foci. The recovery of organisms of suitable antigenicity may not always coincide with clinical expectations.

The cultural procedure may be inadequate from the standpoint of the technique of isolation, the method of selecting the colonies, the incubation period, taxonomic considerations, and the methods used to differentiate the etiologic bacteria from contaminants.

The culture selected may not possess suitable antigenic properties. The antigen may be injured during the cultivation process or in the later manipula-

tion. The injured antigen may contain an excessive ratio of protein to specific antigen, causing it to be toxic and nonspecific.

The dosage may be unsuitable. The injections may be given too often or the amounts may be too large. In other instances the doses may be too small for adequate stimulation of antibodies. The use of a fixed schedule is condemned.

The condition of the patient may not be favorable for optimum antibody response because of endocrine or nutritional deficiencies, liver dysfunction, an excessive toxic load (e.g., an untreated focus), or other factors.

REFERENCES

1. Siler, J. F., Dunham, G. C., Longfellow, D., and Luippold, G. F.: Immunization to Typhoid Fever. Monograph series No. 17, Am. J. Hyg., 1941.
2. Stiles, M. H., and Chapman, G. H.: Probable Pathogenic Streptococci and Staphylococci in Chronic Low Grade Illness. An Analysis of Their Frequency in Three Hundred and Ninety-five Cases, Arch. Otolaryngol. 31: 458, 1940.
3. Ruggier, J. C., and Chapman, G. H.: Newer Bacteriologic Considerations of Dental Infections as Factors in Systemic Disease, J. Tenn. State Dental Assn. 21: 149, 1941.
4. Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. F.: Penicillin as a Chemotherapeutic Agent, Lancet 2: 226, 1940.
5. Abraham, E. P., Chain, E., Fletcher, C. M., Florey, W. H., Gardner, A. D., Heatley, N. G., and Jennings, M. A.: Further Observations on Penicillin, Lancet 2: 177, 1941.
6. Chapman, G. H., Lieb, C. W., and Curcio, L. G.: The Use of Bromthymol Blue Agar and Phenol Red Mannitol Agar for the Isolation of Pathogenic Types of Staphylococci, Am. J. Clin. Path., Tech. Suppl. 2: 3, 1938.
7. Chapman, G. H.: Specificity of Selective Isolation Media for Probable Pathogenic Staphylococci, J. Bact. 43: 105, 1942.
8. Stiles, M. H., and Chapman, G. H.: Selective Isolation of Pathogenic Types of α- and γ-Hemolytic Streptococci, J. Bact. 43: 61, 1942.
9. Chapman, G. H., Berens, C., Nilson, E. L., and Curcio, L. G.: The Differentiation of Pathogenic Staphylococci from Nonpathogenic Types, J. Bact. 35: 311, 1938.
10. Chapman, G. H., Berens, C., and Stiles, M. H.: The Coagulation of Plasma by Staphylococci, J. Bact. 41: 431, 1941.
11. Chapman, G. H., and Stiles, M. H.: The Isolation and Testing of Probable Pathogenic Staphylococci, J. Bact. 39: 5, 1940.
12. Short, C. L., Dienes, L., and Bauer, W.: Autogenous Vaccines in Rheumatoid Arthritis. A Clinical Study and Critique, Am. J. M. Sc. 187: 615, 1934.
13. Chapman, G. H., Berens, C., Lieb, C. W., Rawls, W. B., and Stiles, M. H.: Examination of Cultures From Persons Suspected of Having Chronic Infection, Am. J. Clin. Path. 9: 491, 1939.
14. Chapman, G. H., Berens, C., and Nilson, E. L.: Studies of Streptococci III. Preliminary Attempts to Correlate Resistance to Chemicals etc., With Pathogenic Effects, J. Bact. 31: 338, 1936.
15. Chapman, G. H., Berens, C., Peters, A., and Curcio, L.: Coagulase and Hemolysin Tests as Measures of the Pathogenicity of Staphylococci, J. Bact. 28: 343, 1934.
16. Chapman, G. H., Stiles, M. H., and Berens, C.: The Isolation and in Vitro Testing of Pathogenic Types of Non-Exotoxic Streptococci, Am. J. Clin. Path. 3: 20, 1939.
17. Chapman, G. H., and Lieb, C. W.: Bacteriology of the Intestinal Tract in Certain Disease. II. The Possible Inhibition of Colon Bacilli by Pathogenic Streptococci and Staphylococci, Rev. Gastroenterol. 5: 234, 1938.
18. a. Rawls, W. B., Ressa, A. A., Gruskin, B., and Gordon, A. S.: Thyroid Activity in Chronic Arthritis, Ann. Int. Med. 11: 1401, 1938.
    b. Rawls, W. B.: The Thyroid in Chronic Arthritis, Rheumatism 1: 11, 1939.
19. Stiles, M. H.: Basal Metabolic Rate in Low Grade Chronic Illness, Am. J. Clin. Path. 11: 871, 1941.
20. a. Stiles, M. H.: Desiccated Thyroid in the Treatment of Low Grade Chronic Illness in Children, Arch. Pediat. 59: 749, 1942.
    b. Stiles, M. H.: Hypothyroidism as a Factor in Low Grade Chronic Illness. In preparation.
21. a. Rawls, W. B., Weiss, S., and Collins, V. L.: Liver Function in Rheumatoid (Chronic Infectious) Arthritis. Preliminary Report, Ann. Int. Med. 10: 1021, 1937.
    b. Rawls, W. B., Weiss, S., and Collins, V. L.: Liver Function in Rheumatoid (Chronic Infectious) Arthritis, Ann. Int. Med. 12: 1455, 1939.

22. a. Stiles, M. H., Stiles, M. T., and Kolb, A. McM.: Bromsulphalein Retention in Low-Grade Chronic Illness, J. LAB. & CLIN. MED. 28: 180, 1942.
b. Stiles, M. H.: Low Grade Hepatitis. In preparation.
23. Chapman, G. H.: Bacteriologic Aids in the Diagnosis of Low Grade Chronic ("Focal") Infection. Unpublished studies.

TREATMENT OF EXPERIMENTALLY INDUCED TYPE I PNEUMOCOCCUS PNEUMONIA IN ALBINO RATS*

A COMPARATIVE STUDY OF THE THERAPEUTIC EFFICIENCY OF VARIOUS SULFONAMIDES AND SPECIFIC RABBIT ANTIPNEUMOCOCCUS SERUM AND COMBINATIONS OF THE TWO

ELMER H. LOUGHLIN, M.D., RICHARD H. BENNETT, M.D.,
MARY E. FLANAGAN, B.S., AND SAMUEL H. SPITZ, M.D.,
BROOKLYN, N. Y.

GROSS and Cooper,[1] in 1937, treated Type I pneumococcal infections in rats with sulfanilamide and rabbit antipneumococcal serum. They found that sulfanilamide was better than serum, and that when the two were combined they were more effective than either therapy alone.

In 1939, Kepl and Gunn[2] similarly treated Type I pneumococcal infections in rats with sulfapyridine and specific rabbit serum. They concluded that when serum was begun within four hours after inoculation, the combined use of serum and sulfapyridine proved more effectual than when either was used alone. When infection was well established, however, serum was more efficacious than sulfapyridine, and at this stage the combination of serum and sulfapyridine produced results no better than when serum was given alone.

In 1940, Wright and Gunn[3] reported on the treatment of experimental Type III lobar pneumonia in rats with sulfapyridine and serum. Their results differed somewhat from those of Kepl and Gunn. Wright and Gunn found that, when the infecting dose of Type III pneumococci was relatively small, serum and sulfapyridine were equally efficient, but that when the infecting dose was large, producing a mortality rate of 100 per cent in control animals, the mortality rates with sulfapyridine were lower than those obtained with serum. A combination of serum and sulfapyridine did not reduce the mortality below that obtained with sulfapyridine alone.

In each of these studies the animals were inoculated intrabronchially with pneumococci suspended in gastric mucin. None, however, report on blood cultures prior to and during treatment of the animals, making it difficult therefore to evaluate accurately the effectiveness of the therapy used, except from a standpoint of gross mortality rates. The lack of reports on the results of blood

*From the Department of Internal Medicine Long Island College of Medicine.

Received for publication, February 1, 1943.

The sodium sulfathiazole used in this study was donated by the Winthrop Chemical Company. The dimethyl sulfadiazine used in this study was donated by the Nepera Chemical Company.

cultures makes it impossible to determine how rapidly the therapy was able to dispose of the bacteremic state in those animals which survived.

In 1941,[4] we reported a study of the comparative efficacy of sulfapyridine and rabbit serum when used alone and in combination in treating artificially induced Type I pneumococcal infections in albino rats. We found that in these infections, which, during the animal's life, were manifested by positive blood cultures, type specific serum appeared to be more potent than sulfapyridine alone; whereas, a combination of sulfapyridine and serum, at approximately one-half the dose of either used separately, gave results similar to those obtained with serum alone.

The present report concerns a similar study made to determine the comparative effectiveness of other sulfonamide drugs when used alone or combined with specific antipneumococcal serum in the treatment of Type I pneumococcal infection in albino rats. The sulfonamide drugs employed were sulfathiazole, sodium sulfathiazole, sulfadiazine, sodium sulfadiazine, and dimethyl sulfadiazine. The last-mentioned drug, although used effectively in the treatment of pneumococcal pneumonia in human beings during the past year in England under the name of sulfamethazine,[5] is new to the field of chemotherapy in this country.

METHODS

Inoculation of the rats was accomplished by spraying small amounts of a pneumococcal culture into both the pharynx and nares, a method described in a previous paper.[6] The culture, which was suspended in 15 c.c. of a physiologic solution of sodium chloride, consisted of the washings of the peritoneal cavity of a white mouse infected with Type I pneumococci (R1 strain), and contained, as a result of the peritoneal inflammation, natural mucin in addition to the other components of an inflammatory exudate. The procedure used to inoculate the animals was entirely nontraumatizing, and, we believe, simulated the manner in which human pneumococcal infections occur.

A total of 137 rats, weighing approximately 300 grams each, were thus inoculated. Of these, 125 animals (91 per cent) became infected as manifested by blood cultures positive for Type I pneumococci. Only those animals developing positive blood cultures were used in this study.

Cultures of the blood were made just prior to the initial treatment, at 24 hours after inoculation, and at 48 hours, and 72 hours, just prior to succeeding treatments. They were then made at daily intervals until death ensued or until no growth was obtained. The blood for culture was plated on Avery's medium for pneumococci, to which was added 5.0 mg. per 100 c.c. of paraminobenzoic acid.

All the animals used, whether dying spontaneously before the fifteenth day or sacrificed on the fifteenth day, were autopsied.

Although 125 animals were successfully infected, 114 were included in this report, since in 11 animals the suspensions or solutions of the sulfonamide drugs were introduced accidentally into the trachea, producing immediate death. There was, therefore, an unequal distribution of animals in some of the groups.

To insure accurate dosage, the sulfonamide compounds, either suspended or dissolved in distilled water, were administered by gavage. The antipneumococcal serum was administered intraperitoneally by injection.

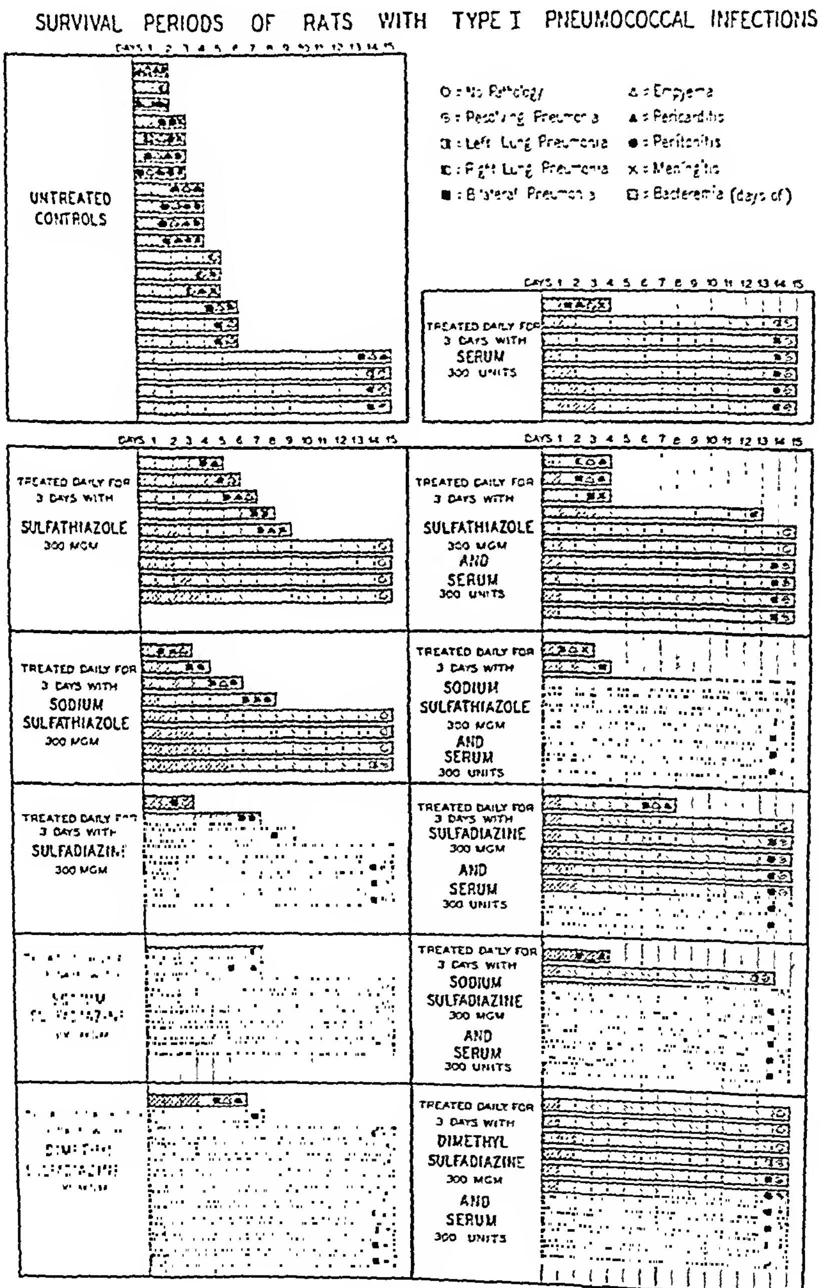


Chart I.

DOSAGE OF RABBIT ANTIPNEUMOCOCCAL SERUM AND THE SULFONAMIDES

A series of 21 animals, in which no therapy had been given, was used as a control for those receiving treatment. Therapy with serum, sulfonamides, and

combinations of the two was begun at twenty-four hours after inoculation with pneumococci.

Serum.—Three hundred units of concentrated Type I rabbit antipneumococcal serum were given on three successive days to each animal of a second series.

Sulfonamides Alone.—To a third series, 300 mg. of a sulfonamide drug per animal (sulfathiazole, sodium sulfathiazole, sulfadiazine, sodium sulfadiazine, or dimethyl sulfadiazine) was given alone to each of five groups of animals on three successive days.

Sulfonamide and Serum Combined.—A combination of 300 mg. of one of the sulfonamides and 300 units of antipneumococcal serum per animal was administered simultaneously daily for three days to each of five groups of rats of a fourth series.

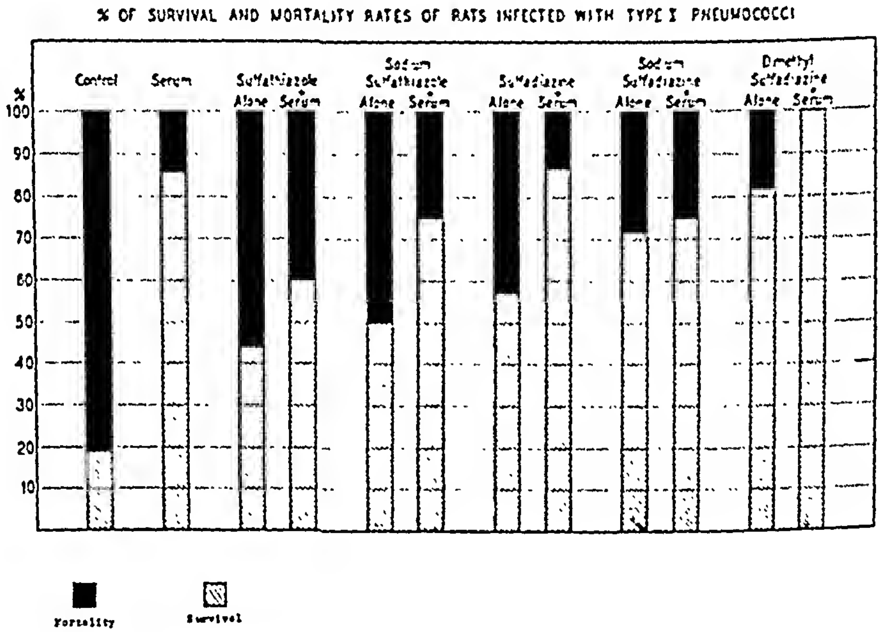


Chart II.

RESULTS

Series I, Control Animals.—Seventeen of the twenty-one rats in this series died. The mortality rate was 81 per cent. The average period of survival of those rats dying spontaneously was 3.9 days. Of these, two had pneumonia alone. Three animals had, in addition to pneumonia, one complication; four had two complications; six had three; and two had four complications. Of the four animals surviving fifteen days and sacrificed, three had resolving pneumonia, while one had bilateral pneumonia complicated by empyema and pericarditis. The average duration of bacteremia of the surviving animals was two days.

Series II, Serum Alone.—One of the seven animals in this series died, giving a mortality rate of 14.3 per cent. This rat, which died spontaneously on the fourth day, had bilateral pneumonia, complicated by empyema, pericarditis,

TABLE I

	NUMBER OF RATS			MORTALITY RATE	AVER. PERIOD OF SURVIVAL OF THOSE DYING SPONTANEOUSLY	AVER. DURATION OF BACTEREMIA OF SACRIFICED RATS
	TOTAL	SURVIVING 15 DAYS	SPONTANEOUS DEATHS	PER CENT	DAYS	DAYS
Series I—Controls	21	4	17	81.0	3.9	2.0
Series II—Serum alone	7	6	1	14.3	4.0	2.0
Series III—Chemotherapy alone						
Group "A"—Sulfathiazole	9	4	5	55.6	7.0	3.0
Group "B"—Sodium sulfathiazole	8	4	4	50.0	5.3	3.5
Group "C"—Sulfadiazine	7	4	3	42.9	6.3	2.3
Group "D"—Sodium sulfadiazine	7	5	2	28.6	9.0	3.4
Group "E"—Dimethyl sulfadiazine	11	9	2	18.2	4.5	1.2
Series IV—Sulfonamide and serum						
Group "A"—Sulfathiazole and serum	10	6	4	40.0	5.5	1.2
Group "B"—Sodium sulfathiazole and serum	8	6	2	25.0	3.5	1.7
Group "C"—Sulfadiazine and serum	8	7	1	12.5	8.0	2.0
Group "D"—Sodium sulfadiazine and serum	8	6	2	25.0	9.0	2.3
Group "E"—Dimethyl sulfadiazine and serum	10	10	0	0		1.6

and meningitis. All those sacrificed had resolving pneumonic processes. The average duration of bacteremia of those sacrificed was two days.

Series III, Chemotherapy Alone.—Group "A" Sulfathiazole.—Five of nine rats died. The mortality rate was 55.6 per cent. The average period of survival of those dying spontaneously was seven days. Three had but one complication; while two had two complications. The four animals which survived for fifteen days had no pathology at autopsy. The average duration of bacteremia was three days in the sacrificed animals.

Group "B" Sodium Sulfathiazole.—Four of eight rats in this group died, the mortality rate being 50 per cent. The average period of survival of those dying spontaneously was 5.3 days. Two had pneumonia with one complication and two had two complications. Three of those sacrificed at fifteen days had no pathology, while one had resolving pneumonia. The average duration of bacteremia of the sacrificed animals was 3.5 days.

Group "C" Sulfadiazine.—Three of seven rats died. The mortality rate was 42.9 per cent. The average period of survival of those dying spontaneously was 6.3 days. Each of these animals had but one complication in addition to pneumonia. Of the four animals sacrificed on the fifteenth day, one had no pathology, while three had resolving pneumonia. The average duration of bacteremia of the sacrificed rats was 2.25 days.

Group "D" Sodium Sulfadiazine.—There were seven animals in this group. Two died; each on the seventh day, giving a mortality rate of 28.6 per cent. One had pneumonia alone; the other had pneumonia complicated by empyema and pericarditis. Four of the five animals sacrificed on the fifteenth day had no

pathology, while one had resolving pneumonia. *The average duration of bacteremia of those sacrificed was 3.4 days.*

Group "E" Dimethyl Sulfadiazine.—Of 11 animals treated, two died, one on the sixth and one on the seventh day, giving a mortality rate of 18.2 per cent; these had respectively bilateral pneumonia, empyema, and pericarditis; and bilateral pneumonia. Of the nine animals which were sacrificed on the fifteenth day, four had no pathology and five had resolving pneumonia. The average duration of bacteremia of the sacrificed animals was 1.22 days.

Series IV, Sulfonamide and Serum Combined.—Group "A" Sulfathiazole and Serum.—There were ten rats in this series. Four, or 40 per cent, died, three on the fourth day and one on the thirteenth day. The average period of survival was 5.5 days. The latter had bilateral pneumonia alone; of the other three, two had pneumonia, empyema, and pericarditis, and one had pneumonia and meningitis. Four of the six rats sacrificed on the fifteenth day had resolving pneumonia, and two had no pathology. The average duration of bacteremia of the sacrificed animals was 1.17 days.

Group "B" Sodium Sulfathiazole and Serum.—Two (25 per cent) of eight rats in this series died on the third and fourth days respectively after inoculation. The former had bilateral pneumonia, empyema, and meningitis; the latter, bilateral pneumonia. The mortality rate was 25 per cent. Of the six animals surviving fifteen days and sacrificed, two had no pathology and four had resolving pneumonia. The average duration of bacteremia of those sacrificed was 1.7 days.

Group "C" Sulfadiazine and Serum.—One of eight rats in this group died, giving a mortality rate of 12.5 per cent. This animal, which died on the eighth day, had bacteremia for one day, and at autopsy, had bilateral pneumonia, empyema, and pericarditis. The seven animals sacrificed on the fifteenth day, with one exception having no pathology, had resolving pneumonia. The average duration of bacteremia of those sacrificed was two days.

Group "D" Sodium Sulfadiazine and Serum.—Two of eight rats in this group died, the mortality rate being 25 per cent. These animals died on the fourth and fourteenth days and respectively had pneumonia, empyema, and pericarditis; and resolving pneumonia. One had no pathology. The average duration of bacteremia of those sacrificed was 2.33 days.

Group "E" Dimethyl Sulfadiazine and Serum.—Ten rats were treated and none died. All ten rats were sacrificed on the fifteenth day. Seven at autopsy had resolving pneumonia, while three had no pathology. The average duration of bacteremia was 1.6 days.

There were no instances of toxicity resulting from any of the therapeutic substances used.

SUMMARY AND CONCLUSIONS

1. Type I pneumococcal infection as manifested by positive blood cultures was produced in 114 albino rats. In those rats dying spontaneously, pneumonia, and in most instances such complications as empyema, pericarditis, peritonitis, or meningitis, were found at post-mortem examination.

2. Sulfathiazole, sodium sulfathiazole, sulfadiazine, sodium sulfadiazine, dimethyl sulfadiazine, and concentrated refined Type I rabbit antipneumococcal serum were used alone and in combination and administered to 93 animals, treatment being started at twenty-four hours after inoculation.

3. In Type I pneumonia in rats, treatment with serum alone and dimethyl sulfadiazine alone resulted in higher survival rates than with any of the other sulfonamides when used alone; whereas, a combination of serum and the sulfonamides gave better results in all instances than the respective sulfonamide used alone. Dimethyl sulfadiazine in combination with Type I serum resulted in higher survival rates than did any of the other sulfonamides. Sulfadiazine combined with serum appeared to be next in effectiveness, while sodium sulfathiazole, sodium sulfadiazine, and sulfathiazole, each combined with serum, were less effectual in this order in saving animals.

When compared with dimethyl sulfadiazine alone, sodium sulfadiazine, sulfadiazine, sodium sulfathiazole, and sulfathiazole were less effective in this order in producing survivals.

4. Sulfathiazole and sulfadiazine were more efficacious in disposing of bacteremia than were their respective sodium salts.

5. Dimethyl sulfadiazine, when used alone, cleared the blood stream of bacteremia more rapidly than any of the other sulfonamides alone, serum alone, or the sulfonamides combined with serum.

We wish to express our appreciation to Doctors Tasker Howard, Wade Oliver, and Arnold Eggerth for their careful criticisms and suggestions in this study.
We wish to thank also Mr. Sidney M. Karlton for his cooperation.

REFERENCES

1. Gross, P., and Cooper, F. B.: P-Aminobenzene-Sulfonamide and Antipneumococcal Serum Therapy in Type I Pneumococcal Infections of Rats, Proc. Soc. Exper. Biol. & Med. 36: 537, 1937.
2. Kepl, M., and Gunn, F. D.: Sulfapyridine and Serum Therapy in Experimental Lobar Pneumonia of Rats, Proc. Soc. Exper. Biol. & Med. 40: 529, 1932.
3. Wright, J. L., and Gunn, F. D.: Sulfapyridine and Serum in Experimental Type III Lobar Pneumonia, Proc. Soc. Exper. Biol. & Med. 44: 523, 1940.
4. Spitz, S. H., Loughlin, E. H., and Bennett, R. H.: Treatment of Pneumonia in Rats, J. Lab. & Clin. Med. 26: 1284, 1941.
5. Macartney, D. W., Smith, G. S., Luxton, R. W., Ramsay, W. A., and Goldman, J.: Sulfamethazine—Clinical Trial of a New Sulphonamide, Lancet 1: 639, 1942.
Jennings, P. A., and Patterson, W. H.: Sulphamethazine—Clinical Trials in Children, Lancet 2: 308, 1942.
6. Loughlin, E. H., Spitz, S. H., and Bennett, R. H.: A Simple Nontraumatizing Method of Inducing Pneumococcic Infection in Albino Rats, J. Lab. & Clin. Med. 26: 1181, 1941.

CLINICAL CHEMISTRY

SIMULTANEOUS PERFORMANCE OF WELTMANN SERUM COAGULATION TEST, CEPHALIN FLOCCULATION TEST, AND MODIFIED TAKATA-ARA REACTION AS AN AID IN THE DIFFERENTIAL DIAGNOSIS OF LIVER DISEASE*

M. WACHSTEIN, M.D., MIDDLETOWN, N. Y.

AMONG the many procedures devised for the correct diagnosis of liver diseases, those which can be performed without too much inconvenience to the patient and which can be done without too much technical difficulty are of definite advantage. Takata-Ara reaction, cephalin flocculation test, and Weltmann serum coagulation test can be performed in *one serum sample* in a fairly simple way. It is the intention of this paper to show that the simultaneous use of all three tests may be helpful in arriving at a correct differential diagnosis in a patient suffering from disease of the liver.

The Weltmann serum coagulation reaction was performed according to the original paper of Weltmann.[1, 2] The stock solution of 5 per cent calcium chloride is best prepared by dissolving 6.7 Gm. calcium chloride .2 H_2O in 100 c.c. distilled water. The final solutions which are prepared from this stock solution are numbered from one to ten. A solution 7½, containing 0.35 c.c. stock solution in 100 c.c. water, is also used. A clearly recognizable coagulation appears in the first 6 or 7 test tubes in sera from normal individuals after the test tubes containing the final dilutions of $CaCl_2$ and serum have been boiled for fifteen minutes. Under abnormal conditions, coagulation may occur in fewer than 6 test tubes, and the coagulation band, as it is called by Weltmann, is shortened or shifted to the left. Or there is coagulation in more than 7 test tubes, and the coagulation band is lengthened or shifted to the right.

Takata-Ara Reaction.—The Takata-Ara reaction was performed in the modification of Mancke and Sommer.[3] By using this method the amount of serum is kept constant, but the concentration of mercuric chloride is varied. The advantage of using this modification is the possibility of grading the reaction in a fairly simple way. No flocculation or flocculation up to the second tube constitutes a negative reaction. Flocculation up to the third or fourth tube is considered a weak positive, and flocculation beyond the fourth tube is considered a strong positive reaction.

Cephalin Flocculation Test.—The cephalin test[4, 5] was performed with the commercially obtainable Difco product. According to Mateer and co-workers,[6] this product, which is made of an especially ripened cephalin, gives reliable results. Doubtful reactions as well as weak positive ones were not regarded as positive.

---

*From the laboratories of the Elizabeth A. Horton Memorial Hospital in Middletown, N. Y., and the Beth Israel Hospital in Passaic, N. J.

Received for publication, May 28, 1943.

Fifty-two cases of primary liver disease or presenting jaundice and 70 control cases were used. The liver cases were divided into three groups: cirrhosis of the liver, diffuse hepatitis, and obstructing jaundice. Only those cases were used in which the diagnosis was reasonably certain. Cases not suffering from liver disease were used as controls.

Results.—(a) Diffuse Hepatitis.—In one of the 13 cases of diffuse hepatitis the Weltmann test showed a slight shortening of the coagulation band, in three cases a slight prolongation, and in 9 cases a marked prolongation. It is noteworthy that the most pronounced shift to the right of the coagulation band was observed in this group. (In 3 cases coagulation included dilution 9.) The Takata-Ara reaction was marked positive in 3 cases, slightly positive in one case, and negative in the remaining 9 cases. The cephalin test was positive in 7 out of 13 instances.

(b) Obstructive Jaundice.—Twenty-six cases of obstructive jaundice were examined. In 12 cases the obstruction was due to neoplasm, in 14 cases to stones. In the cases in which the obstruction was due to neoplasm, the coagulation band was in 3 cases shortened, in seven cases normal, and in only 2 cases slightly prolonged (dilution 7½). In the cases of obstructive jaundice due to stones, the Weltmann test showed in four cases a shortening and in 10 cases a normal range. The Takata-Ara reaction was one time strongly and 3 times weakly positive in the cases of obstructive jaundice due to stones; twice strongly and twice weakly positive in the neoplastic group. The cephalin test was three times positive in the stone group and twice positive in the other group, altogether 5 times in 26 cases. Erroneous results of one of the tests are frequently counterbalanced by the correcting results of the other tests. For instance, one case in this group showed a positive cephalin test, but the Takata-Ara reaction was negative and the coagulation band was shortened to 2.

(c) Cirrhosis.—In 14 cases of cirrhosis, 8 showed a marked and 2 a moderate prolongation of the coagulation band. In four cases the coagulation bands were normal. The Takata-Ara reaction was strongly positive in twelve cases, weakly positive in one case, and negative in one case. The cephalin test was 11 times positive and twice negative. In one case it was several times negative and became positive only a few weeks before the death of the patient.

Control Cases.—In 70 control cases the 3 reactions were simultaneously performed. The Takata-Ara reaction was 11 times strongly positive and 6 times weakly positive. The cephalin test was 6 times positive, once in a case of pneumonia, twice in cases of subacute bacterial endocarditis, once in a case of nephrosis, and once in a case of multiple myeloma. The results of the Weltmann test varied in different conditions. The usefulness of this test in the interpretation of various diseases has been confirmed by Teufl,[7] Levinson, Klein, and Rosenblum,[8] Levinson and Klein,[9] Dees,[10] and Kraemer.[11] Two cases of subacute bacterial endocarditis showed a lengthening of the coagulation band, a positive cephalin test, and a negative Takata-Ara reaction. These findings suggested diffuse hepatitis. There was no jaundice present, however, and there was no reason to doubt the diagnosis of subacute bacterial endocarditis on clinical and bacteriologic grounds. In one other patient suffering from cardiac failure, the results of the three tests suggested a complicating cirrhosis. The

autopsy revealed, however, only chronic congestion of the liver. In all other control cases the results of the combined tests were not misleading.

Comment.—The simultaneous use of the 3 reactions greatly enhances their value as can be recognized by critical evaluation of the tests. None of the reactions are specific. Magath[14] concludes on the basis of the literature as well as on his own extensive experience that the Takata-Ara reaction may give positive results in a wide variety of diseases. It is not a specific test but gives usually a positive result in more advanced cases of liver cirrhosis. It is frequently positive in acute hepatitis and malignant disease of the liver. The use of the cephalin flocculation test to demonstrate active disease of liver parenchyma is, without question, very valuable. We found, however, as in the Takata-Ara reaction, a number of wrong positives (8.6 per cent). A positive cephalin test might, therefore, occasionally be misleading in a patient with questionable liver disease without jaundice. The test does not always allow a differentiation between hepatogenous and obstructive jaundice. In 13 cases of hepatogenous jaundice we found the test positive only 7 times. On the other hand, the test was positive 5 times in 26 cases of obstructing jaundice. In our series the highest number of positive reactions was found in the group of liver cirrhosis. The Weltmann reaction showed the most pronounced prolongation in cases of diffuse hepatitis and also very marked prolongation in cirrhosis. It was usually normal or shortened in cases of obstructing jaundice. These findings are in full agreement with the experience of Weltmann and Sieder,[13] Teufl,[7] and Levinson and Klein.[8] The tendency to the lengthening of the coagulation band is very strongly maintained in cirrhosis especially. Complicating processes, which otherwise would lead to a marked shortening of the band, have slight, if any, influence. The coagulation band is an unspecific reaction, however, and marked lengthening is occasionally seen in other conditions.

Summary and Conclusion.—Weltmann serum coagulation band, cephalin flocculation test, and Takato-Ara reactions in the modification of Mancke and Sommer were performed. In 52 liver cases and 70 control cases it was found that by using all 3 simple tests in the same serum sample, their value for the differentiation of liver diseases could be markedly increased. Thirteen cases of diffuse hepatitis showed a frequent and pronounced prolongation of the coagulation band, a frequently positive cephalin test, and only an occasional positive Takata-Ara reaction. In 26 cases of obstructing jaundice there was found either a shortened or normal coagulation band, an occasional positive Takota-Ara reaction, and only an occasional positive cephalin test. In 14 cases of cirrhosis of the liver a prolongated coagulation band, a positive Takata-Ara reaction, and a frequently positive cephalin test were found. Only in one out of 70 control cases did the clinical picture and result of the tests lead to the erroneous diagnosis of cirrhosis of the liver.

REFERENCES

1. Weltmann, O.: Ueber die Spiegelung Exudativ-entzuendlicher und Fibroeser Vorgaenge im Blutserum, Med. Klin. 26: 240, 1930.
2. Weltmann, O.: Zur Leber Pathologie, Wien. klin. Wchnschr. 43: 1301, 1930.
3. Mancke, R., and Sommer, J.: Die Abgestufte Takata-Reaktion im Serum zur Diagnose von Leberkrankheiten, München. med. Wchnschr. 2: 1707, 1936.
4. Hanger, F. M.: The Flocculation of Cephalin Cholesterol Emulsions by Pathological Sera, Tr. A. Am. Physicians 53: 148, 1938.

5. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculations of Cephalin Cholesterol Emulsions, J. Clin. Investigation 18: 261, 1939.
6. Mateer, J. G., Baltz, J. I., Marion, D. F., MacMillian, J. M.: Liver Function Tests, J. A. M. A. 121: 723, 1943.
7. Teufl, R.: Der Diagnostische Wert der Serum Koagulation, Wien. Arch. f. inn. Med. 28: 305 and 320; 29: 37 and 297, 1935.
8. Levinson, S. A., Klein, R. I., and Rosenblum, P.: Weltmann Serum Coagulation Reaction, J. Lab. & Clin. Med. 23: 53, 1937.
9. Levinson, S. A., and Klein, R.: The Value of the Weltmann Se[illegible] Reaction as a Laboratory Diagnostic Aid. Comparison With the [illegible] Ann. Int. Med. 12: 1948, 1939.
10. Dees, S. C.: A Clinical Study of the Weltmann Serum Coagulation Reaction, J. Pediat. 17: 44, 1940.
11. Kraemer, M.: The Serum Coagulation Reaction. Its Clinical Significance, Amer. J. Digest. Dis. 9: 129, 1942.
12. Magath, Th. B.: The Takata-Ara test in Liver Disease, J. Lab. & Clin. Med. 26: 156, 1940.
13. Weltmann, O., and Sieder, B.: Die Bedeutung des Koagulations-Bandes fuer die Diagnose der Leberkrankheiten, Wien. Arch. f. inn. Med. 24: 321, 1934.

PREPARATION AND PROPERTIES OF A DRY POWDERED MIXTURE OF SULFANILAMIDE AND HEMOSTATIC GLOBULIN*

I. A. Parfentjev, Ph.D., Mary A. Goodline, B.A., and Frances L. Clapp, B.A., Pearl River, N. Y.

About two years ago, we isolated from rabbit plasma a fraction possessing a high capacity to clot fibrinogen.[1,2] Solutions of this fraction diluted to a protein content of 10 per cent are stable and exhibit high thrombic activity. The material was preserved with 0.3 per cent phenol and phenyl mercuric acetate 1:20,000. Prepared in this way, hemostatic globulin could be Berkefeld-filtered and stored at 5° C. for over one year, at the end of which time the product showed only a slight loss of potency.[1,2]

Extensive studies on the physiologic action and clinical application of hemostatic globulin were performed by Finland and Taylor and their collaborators.[3,6,7] Additional information was presented in the paper of Bird, McSwain, Kauer, and Glenn, introduced by Reznikoff.[5] Recently Taylor and Adams extended their studies on thrombic activity of a globulin fraction prepared similarly from other species of blood (beef, swine, and human).[4] Ferguson used hemostatic globulin in the estimation of fibrinolysis.[8,9] An editorial in the *Lancet* has reviewed the work so far performed with hemostatic globulin isolated by us from rabbit plasma.[13]

During clinical trial, it was found that these preparations could be diluted to 1 per cent protein (1:10) before being used. Such dilute solutions still contained enough potency to stop, by local application, bleeding in normal persons and in hemophiliacs.[3-5] Studies on the stability of hemostatic globulin in more dilute solutions (than 10 per cent protein) and at different pH levels are now in progress.

*From the Lederle Laboratories, Inc.

Received for publication, June 23, 1943.

Recently, we prepared dry hemostatic globulin by the process (lyophilization) that is used for the drying of human plasma. This dried material was ground in a mortar and sterilized by dry heat at 110° C. for eight hours. The treatment did not affect the solubility of hemostatic globulin. Its thrombic activity was also found to be very little affected by this method of drying and sterilization. Contrary to this, solutions of clotting globulin are much more thermolabile, temperatures high enough for coagulation of proteins rapidly destroying their clotting capacity.

The next step of our research was to prepare a mixture of dry hemostatic globulin and sulfanilamide (U.S.P., 40 to 80 mesh crystals). Hemostatic globulin was added in the amount of 5 per cent to the sulfanilamide. The materials were thoroughly mixed by grinding in a mortar, and the mixture was then sterilized by dry heat at 110° C. for eight hours in small samples, not exceeding 5 Gm. Material prepared by this method was found to be sterile.

For testing the sterility of the powdered mixture, advantage was taken of the fact that bacteriostatic action of sulfa drugs is neutralized by para-aminobenzoic acid.[10-12] The usual liquid medium (Brewer's) employed for sterility tests, and suitable for both aerobic and anaerobic growth, was modified by the addition of 10 mg. per 100 c.c. para-aminobenzoic acid. To approximately 40 c.c. of this medium in Smith fermentation tubes were added by means of a sterile glass spoon approximately 50 mg. of the powdered mixture from each container tested. At least 3 per cent of the containers in each experimental lot were thus tested. The incubation period was seven days at 37° C. To check the broth for its suitability for use in sterility testing of sulfanilamide, one tube inoculated with the powdered mixture was also inoculated with pneumococci. Prompt growth of these cocci resulted upon incubation.

TABLE I

SHOWING THAT MIXTURES OF POWDERED HEMOSTATIC GLOBULIN AND POWDERED SULFANILAMIDE CAN BE STERILIZED WITH DRY HEAT WITH APPARENTLY NO LOSS OF POWDERED PROPERTY

	133H168 ORIGINAL HEMOSTATIC GLOBULIN	*26C2* MIXTURE OF SULFANILAMIDE AND HEM. GLOB. BEFORE STERILIZATION	*26C2* MIXTURE OF SULFANILAMIDE AND HEM. GLOB. AFTER STERILIZATION
DILUTIONS	DEGREE OF CLOTTING (CLOTTING TIME—5 MINUTES)		
1:10	++++	--	--
1:20	++++	--	--
1:40	++++	++++	++++
1:80	+++	+++	+++
1:160	++	++	++
1:320	±	±	±
1:640	±	±	0
1:1280	0	0	0

After sterilization the material was tested for clotting potency. The results of these tests, given in Table I, demonstrate that the thrombic activity of hemostatic globulin in the sterile sulfanilamide mixture remained unimpaired. In order to measure the thrombic activity, 105 mg. of the dry powdered mixture were suspended in 2 c.c. of physiologic saline solution, corresponding to a 1:40 dilution of original hemostatic globulin (10 per cent protein solution). From this suspension doubling dilutions were made. To 0.1 c.c. of each dilution in Wassermann tubes was added 0.9 c.c. of oxalated normal horse

plasma (0.26 per cent) preserved with 0.45 per cent phenol. The tubes were placed in a water bath at 37° C., and the degree of clotting was recorded at the end of five minutes.

In the table the column at the left records the clotting titer of the original hemostatic globulin, No. 133H168, prepared as a solution containing approximately 10 per cent protein and preserved with 0.3 per cent phenol and phenyl mercuric acetate 1:20,000. The middle column records the clotting titer of the mixture of dried hemostatic globulin (prepared from No. 133H168) and sulfanilamide, while the column at the right records the clotting titer of the same mixture after sterilization.

Clots are designated as follows:

++++	Solid clot: adheres to sides when tube is inverted.
+++	Firm clot: does not adhere to sides when tube is inverted.
++	Partial clot: flows along sides when tube is inverted.
+	Small clot: most of the material remains liquid.
±	Strands of fibrin observed when tube is shaken.

In the preceding paragraphs we have described the method of preparation of a sterile mixture of powdered sulfanilamide and hemostatic globulin. Preparation of such a mixture became possible after it was observed that dry hemostatic globulin possessed remarkable stability at high temperatures. As stated above, dry powdered hemestatic globulin withstands eight hours' heating at 110° C., and at the end of this period shows very little loss of its thrombic activity.

SUMMARY

1. Evidence is presented that dry hemostatic globulin possesses remarkable stability at high temperatures, withstanding eight hours' heating at 110° C., at the end of which period it shows very little loss of its thrombic activity.

2. The mixture of hemostatic globulin and sulfanilamide may be of value in the treatment of wounds, since it possesses both thrombic and bacteriostatic properties.

REFERENCES

1. Parfentjev, I. A.: A Clotting Factor in Rabbit Plasma. Science 93: 328, 1941.
2. Parfentjev, I. A.: A Globulin-Fraction in Rabbit's Plasma Possessing a Strong Clotting Property, Am. J. M. Sc. 202: 587, 1941.
3. Lozner, E. L., MacDonald, H., Finland, M., and Taylor, F. H. L.: The Use of Rabbit Thrombin as a Local Hemostatic, Am. J. M. Sc. 202: 593, 1941.
4. Taylor, F. H. L., Lozner, E. L., and Adams, M. A.: The Thrombic Activity of a Globulin Fraction Derived from Rabbit Plasma, Am. J. M. Sc. 202: 585, 1941.
5. Bird, R. M., McSwain, B., Kauer, Jr., G. L., and Glenn, Frank: Clotting Action of Rabbit Clotting Globulin, Proc. Soc. Exper. Biol. & Med. 48: 730, 1941.
6. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L.: Studies on Blood Coagulation: A Proteolytic Enzyme Prepared from Calcium and Platelet Free Normal Human Blood Plasma, J. Clin. Investigation 21: 525, 1942.
7. Tagnon, H. J., and Taylor, F. H. L.: Effect on Coagulation Time of Oral Administration of Rabbit Thrombin, Proc. Soc. Exper. Biol. & Med. 49: 32, 1942.
8. Ferguson, J. H.: Estimation of Trypsin by Fibrinolysis, Proc. Soc. Exper. Biol. & Med. 51: 73, 1942.
9. Ferguson, J. H.: Crystalline Trypsin-Inhibitor and Blood Clotting. Proc. Soc. Exper. Biol. & Med. 51: 373, 1942.
10. Woods, D. D.: The Relation of p-Aminobenzoic Acid to the Mechanism of the Action of Sulfanilamide. Brit. J. Exper. Path. 21: 74, 1940.
11. Janeway, C. A.: Method for Obtaining Rapid Bacterial Growth in Cultures from Patients Under Treatment With Sulfonamides. J. A. M. A. 116: 941, 1941.
12. Roblin, Jr., R. O.: Personal communication. Feb. 1942.
13. Thrombin as a Hemostatic (editorial), Lancet 1: 10, 1942.

LABORATORY METHODS

GENERAL

A SIMPLE METHOD FOR THE CONSTRUCTION OF NOMOGRAPHS*

C. B. Cosby, M.A., and J. H. Weatherby, Ph.D., Richmond, Va.

FREQUENTLY it is desirable to make many rapid calculations of one general type. Nomographs have been devised for this purpose. While many ingenious ways of devising them have been offered, all depending essentially on graphic representation of logarithms, the one described here is believed to embody several distinct advantages. For other methods, as well as a general discussion of nomography, the appended bibliography may be consulted.

Nomographs are not uncommonly seen in such diverse journals as "Barron's Financial Weekly," "Educational Psychology," "Anatomical Record," and the "American Journal of Hygiene." Statisticians and actuaries use them so frequently that there is now available a "Handbook of Statistical Nomographs." Since they are a valuable tool in engineering and scientific research, a simple method for their construction would appear to be of value.

Mathematical formulas are merely expressions of addition, subtraction, multiplication, and division of variables and their constants. In Fig. 1 is shown the scheme for simple addition or subtraction of two numbers, which plan represents the fundamental device of nomography. Here scales are equally divided and evenly spaced apart. Numerical values are assigned to the scales so that any point on the middle scale has twice the value of the corresponding point on either of the other scales. For example, in the addition of 4 and 12, a line called the isopleth line, drawn from 4 on one side scale to 12 on the other side scale, crosses the middle scale at 16. To substract 4 from 16, a line is drawn from 4 on the left scale through 16 on the middle scale until it intersects the right scale, this point of intersection indicating the difference between the two numbers.

Since multiplication and division can be accomplished quite conveniently by the addition or subtraction of logarithms, this same principle can be applied to these processes. When the scales are graduated logarithmically instead of equally, the distances can be added or subtracted, giving the desired multiplication or division. For this purpose the value of any point on the middle scale is made equal to the square of the corresponding point on either of the side scales, as shown in Fig. 2. Since any selected distance on the side scales is

*From the Department of Pharmacology, Medical College of Virginia, Richmond, Va. Richmond, Va.
Received for publication, October 2, 1942.

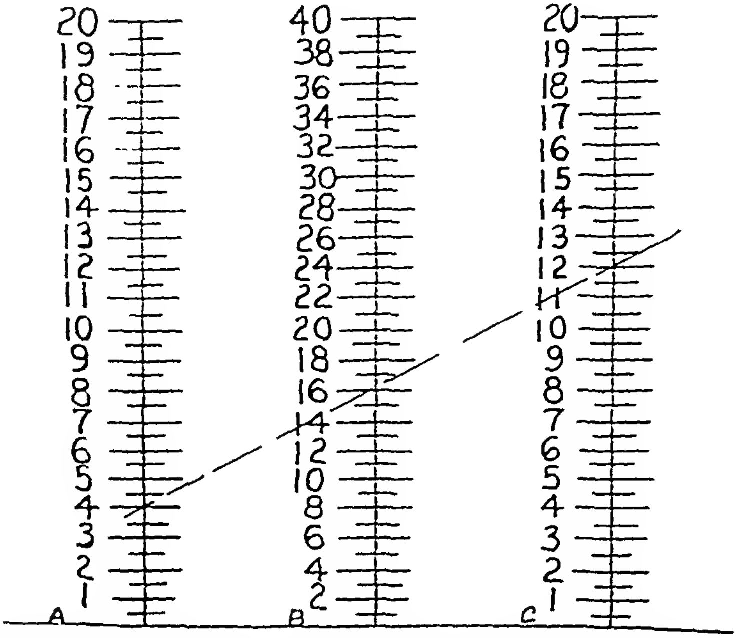


Fig. 1.—Simple nomographic addition. A + C = B, the diagonal line indicating 4 + 12 = 16 or 16 − 4 = 12.

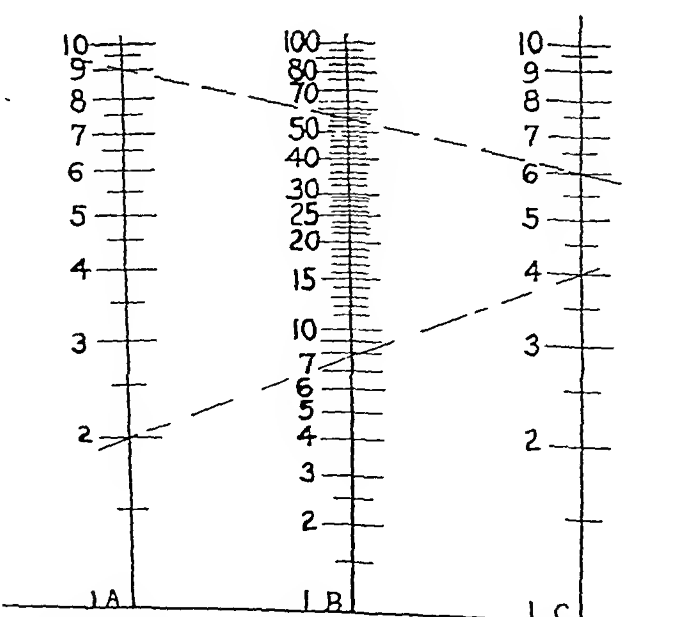


Fig. 2.—Simple multiplication, logarithmic division of scales; the product of A × C = B. As shown, the diagonal lines indicate 9 × 6 = 54 and 8 ÷ 2 = 4.

proportional to the logarithm of a number, the addition of such distances results in multiplication of the numbers. As shown, we have $9 \times 6 = 54$ and $8 \div 2 = 4$. If the side scales are not equally spaced from the middle, this displacement in itself constitutes a factor of multiplication. In the simplified system presented here intentional displacement of this kind is avoided.

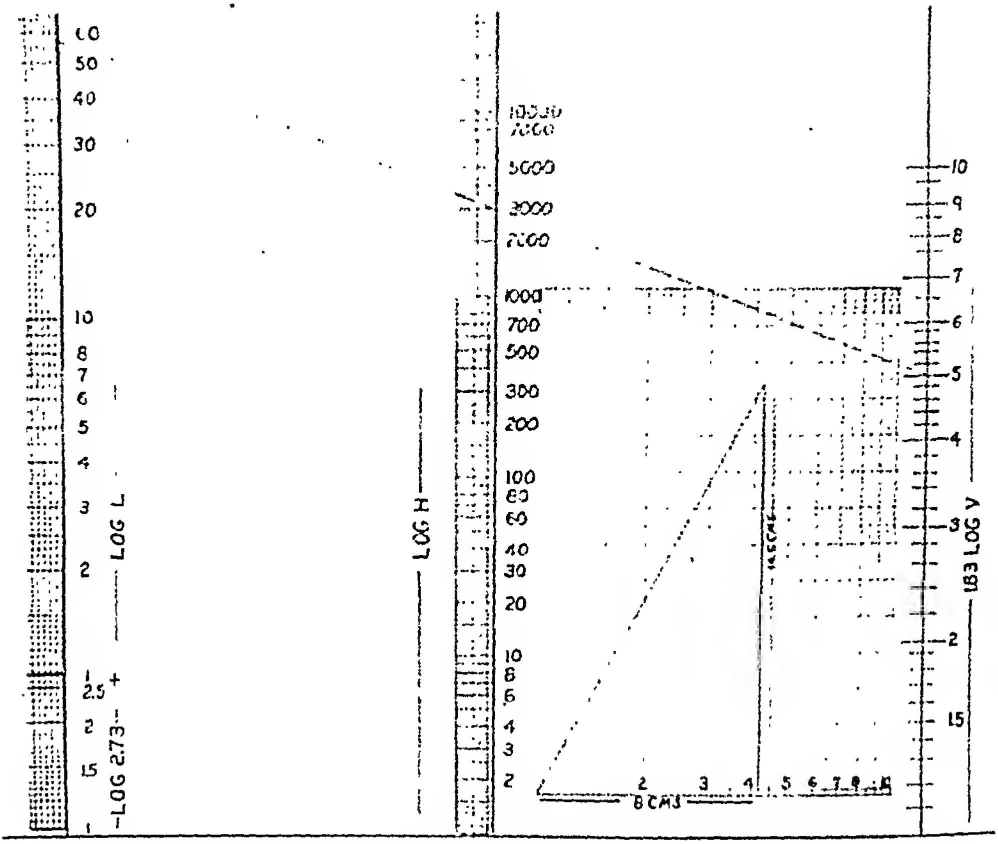


Fig. 3.—A nomograph to show $h = 2.73\ LV^{1.83}$ or $\log h = \log 2.73 + \log L + 1.83 \log V$. h = loss of head pressure in 2" pipe in ft. ÷ 1000, L = length of pipe in ft./1000 and V = velocity of liquid ft./sec.

In Fig. 3 is shown the formula and the nomograph for the calculation of the loss of pressure in a pipe in which the loss is dependent on length and velocity of the liquid passing through the pipe, $H = 2.73\ LV^{1.83}$. The novel feature here consists in the use of strips of logarithm paper held in position on stiff bristol board by cellulose tape. This obviates the laborious and tedious division of scales and also insures greater accuracy. The center scale is equally spaced between the side scales. Further, all scales begin at the same base line of value 1, so that decimals, digits, tens, etc., are easily determined. Usually logarithms are used to solve this equation; i.e., the log of $h = \log 2.73 + \log L + 1.83 \log V$. The log of 2.73 is added to the log of L by cutting a strip of log paper at 2.73, and then the regular cycles of logarithms are placed above so that 1 begins at 2.73. To obtain 1.83 log V, in the insert, 8 cm. were measured off horizontally on logarithm paper, 1.83×8 cm. vertically, and a line was drawn as shown. Each unit of length on the horizontal scale has above it a segment 1.83 times

its length. These values are transferred to the right hand scale by means of dividers. The center scale is of paper twice as finely divided as the left scale. Anyone contemplating constructing nomographs will find the several varieties of log or semilog paper available of great assistance.

In Fig. 4 is a nomograph for calculating the resistance of a parachute, in which the same system has been used. The square of V is obtained by using for the right hand scale paper that is twice as coarse as that used on the left, that is, having one cycle correspond in length to two cycles. For the center scale the paper is twice as finely divided as that used for the left scale.

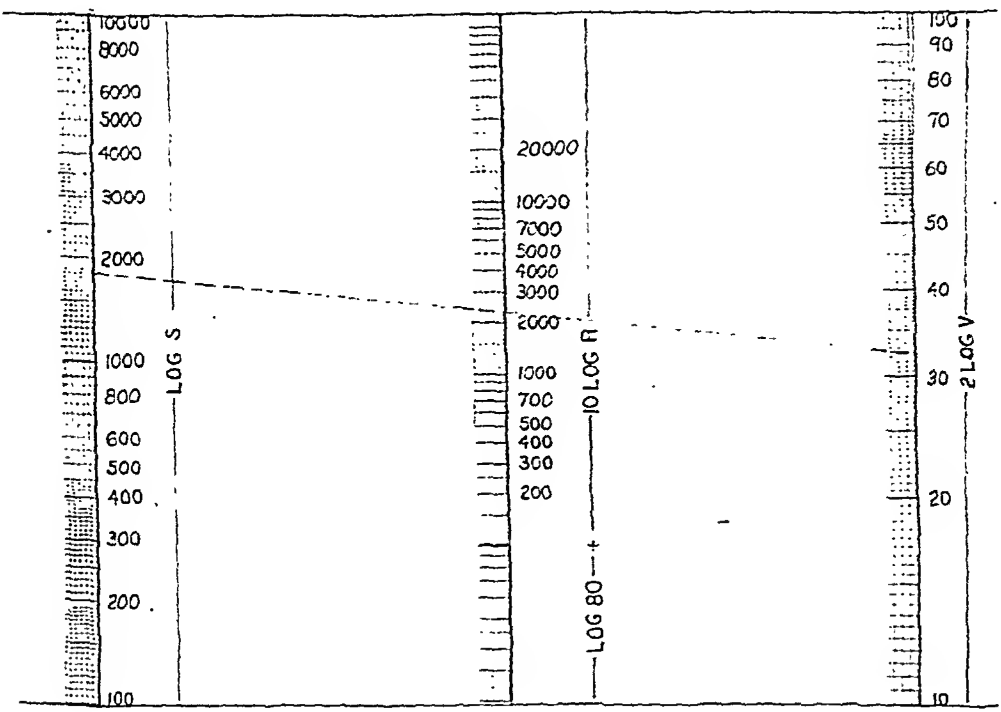


Fig. 4.—A nomograph for the resistance of a parachute as given by the formula $R = \frac{SV^2}{008}$ or 800 $R = SV^2$. R = resistance, S = area in ft.², V = velocity in ft./sec.

In Fig. 5 is shown the formula for the standardization of insulin in which there are five variables. Two variables are combined by division to obtain a reference line, this combined with a third variable to obtain a second reference line, which is left in the middle product position, and to the right is placed the C scale for division by C. Of course the constant 1.5 is taken into account by shifting A upward 1.5 logarithmic units. Finally the remaining scale gives the values of U. It should be noted that the reference lines are not divided, since their purpose is merely to give points for beginning the next operation. The value of these points as such is not needed.

The accuracy of these nomographs, just as in arithmetical multiplication, depends on the number of decimal places one keeps; that is, on logarithm paper one cycle corresponds to one decimal place. Any misplacement of the axes introduces an error. The limit of fine division varies from 25 to 100

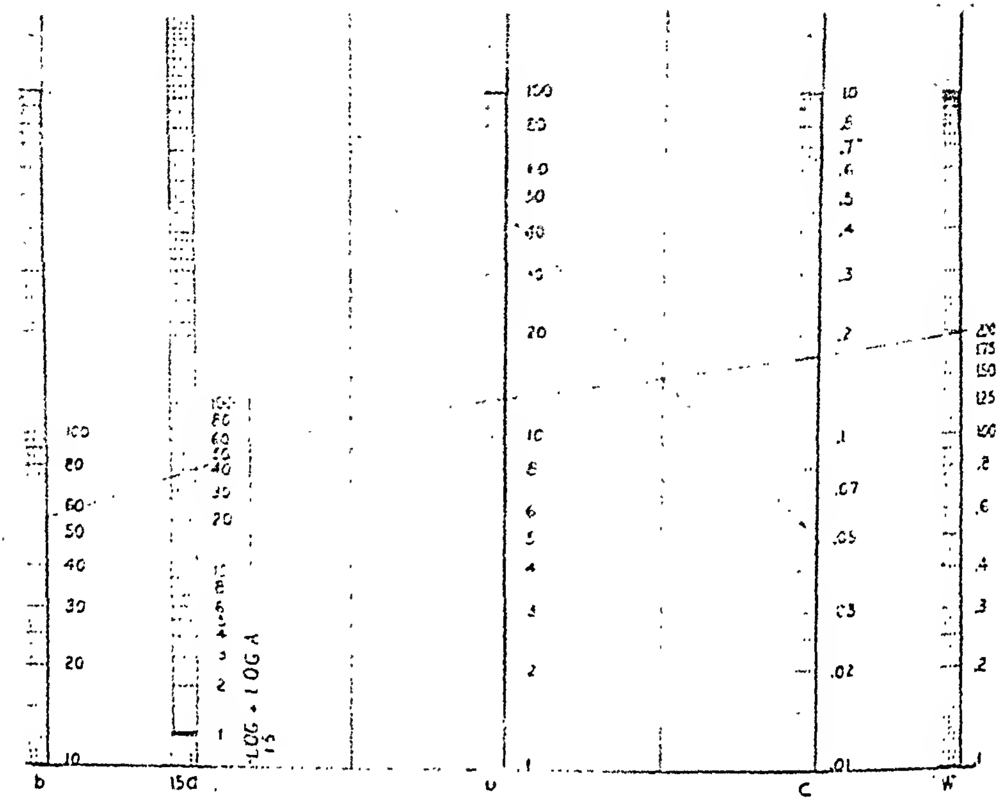


Fig. 5.—A nomograph for the standardization of insulin. $U = \frac{1.5\ AW}{bc}$, W = wt. of rabbit in kg., C = c.c. of injection, a and b, blood sugar percentages as usual in technique.

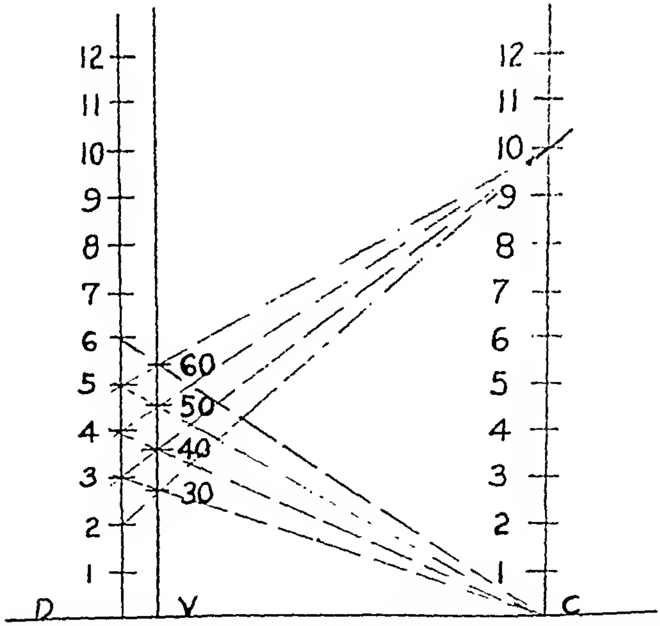


Fig. 6.—Empirical derivation of a nomogram for the simple formula V = 10 d + c, V = value in cents of a mixture of dimes and pennies, D = number of dimes, c = number of pennies.

divisions per inch, depending on the instruments used and the care exercised. Accuracy may be increased by enlarging the graph, but size ultimately reaches a conveniently usable maximum. Also, the isopleth should never slant more than 45 degrees from the horizontal, since intersections are less distinct at a greater angle. Also, it must be remembered that errors are compounded as the process is carried from scale to scale. All these may be minimized by care in construction of the nomograph, by the use of equally spaced axes when possible, having all scales begin at the same base line, and by the use of machine-ruled paper whenever possible. For permanent use and for copies, large photographs may be used under ground glass. The isopleths may then be drawn and sponged off repeatedly.

The nomographs illustrated here were selected for purposes of demonstration only, and not for specific application. While novel effects may be obtained with systems other than that employing vertical equally spaced axes, the ease and simplicity of the procedure outlined are to be recommended.

For unusual situations, such as those involving the functions of angles, recourse may be had to the empirical method as simplified in Fig. 6. Here, the result desired is the number of cents which are equivalent in value to a mixture of dimes and cents. The outer scales are evenly divided. The location of the middle scale, as well as its divisions, is determined in the following manner. Isopleths are drawn from the points zero and ten on the C scale to several points (2, 3, 4, 5) on the D scale. The intersections of the isopleths locate the position of the third scale and its divisions. The outer scales are straight vertical lines; the resulting intersections may indicate a straight line as shown in this illustration, but in other more complex relations, usually a simple curve.

REFERENCES

Allcock, H. J., and Jones, J. R.: The Nomogram, New York, 1938, Pittman Pub. Corp.

Brodetsky, S.: A First Course in Nomography, London, 1920, G. Bell & Sons.

D'Ocagne, M.: Le Calcul Simplifié par les procés des mécaniques et graphiques, Paris, 1894; and Les Calculs usuels affectués au moyen des abaques, Paris, 1891.

Dunlap, J. W., and Kurtz, A. K.: Handbook of Statistical Nomographs, 1932, World Book Co.

Griffith, James R.: Construction Design Charts, San Francisco, 1940, Western Construction Publications.

Haskell, Allan Cecil: How to Make and Use Graphic Charts, New York, 1919, Codex Book Co.

Lehoczky, Paul N.: Alignment Charts, Their Construction and Use, Colnmbus, Ohio, 1936, Ohio State University.

Luckey, Paul: Praktische Anleitung zum entwerfen graphische, Rechentafeln. . . , Berlin, 1937, B. G. Teubner.

Strachen, R. C.: Nomographic Solutions of Formulas of Various Types, Proc. Am. Soc. of Civ. Eng. 40: 2975, 1914.

Swett, Geo. Wright: Construction of Alignment Charts, New York, 1929, Wiley.

Van Voorhes, Merrill G.: How to Make Alignment Charts, New York, 1937, McGraw-Hill.

TWO NEW METHODS OF STAINING VAGINAL SMEARS*

Dorothea Fuller, B.S., M.A., New York, N. Y.

During recent years great interest has developed in studies of the vaginal secretions, based on the work of Papanicolaou. From time to time new staining techniques have been devised.

In this communication two stains† are described. The first, PGI, is recommended for studying the menstrual cycle; the second, Post Graduate II, is designed for the differentiation of malignant cells.

POST GRADUATE I FOR ROUTINE SMEARS

Fix smears immediately in equal parts of 95 per cent ethyl alcohol and ether for at least five minutes.

Run down through graded alcohols (80 per cent, 50 per cent, 30 per cent) to water. Stain in Ehrlich's hematoxylin diluted one-half with distilled water for two minutes. Wash in running water for four minutes.

Stain for two minutes in I.

Rinse quickly in water.

Stain for two minutes in II.

Rinse quickly in water.

Stain for one minute in III.

Rinse quickly in water and run up through alcohols to xylol and mount in clarite or dammar.

Staining Solutions:

1. (a) 1 Gm. ponceau de xylidine.
   99 c.c. distilled water.
   1 c.c. glacial acetic acid.
   (b) 1 Gm. acid fuchsin.
   99 c.c. distilled water.
   1 c.c. glacial acetic acid.
   Mix two parts of (a) with one of (b).
2. 2 Gm. orange G.
   1 Gm. phosphomolybdic acid.
   100 c.c. distilled water.
3. 1 Gm. fast green.
   100 c.c. distilled water.

Superficial cells stain green, deep cells a slightly darker green; highly cornified cells stain orange, and the less cornified cells, pink. Red blood corpuscles, when fresh, stain pink; when old, green.

---

*From the Department of Gynecology, New York Post-Graduate Medical School and Hospital.

These studies were made possible by a grant from Mr. Jeremiah Milbank.

I wish to express my grateful appreciation to Dr. Walter Dannreuther for his constructive criticism.

†All the stains are National Aniline and Chemical Co. dyes.

Received for publication, September 29, 1942.

POST GRADUATE II FOR MALIGNANT CELLS

Fix, and run down to water, and stain in the diluted Ehrlich's hematoxylin as before.

Wash and run up through ascending alcohols (50 per cent, 80 per cent, 95 per cent).

Stain for two minutes in the following stain:

11 c.c. saturated eosin (in 95 per cent alcohol).
35 c.c. saturated orange G (in 95 per cent alcohol).
8 c.c. saturated fast green (in 95 per cent alcohol).

(In mixing this stain, add the eosin to the orange G and shake; then add the fast green slowly.)

Differentiate (agitate vigorously and rapidly) in 99 c.c. absolute alcohol with 1 c.c. glacial acetic acid in it for about one minute. Rinse in absolute alcohol to xylol and mount as before.

(This stain does not keep very long in its mixed form, and the saturated solution of orange G must be the first saturation. If the malignant cells stain too dark, add a few more c.c. of orange G.)

Superficial cells stain green, the cornified cells, red, and the malignant cells are usually red or purple.

These two stains have been used routinely in the endocrine clinic of the Department of Gynecology at the New York Post-Graduate Medical School and Hospital. They have shown a high degree of specificity and have proved eminently satisfactory in cell differentiation and diagnosis.

ON THE USE OF FIREPROOF COTTON IN BACTERIOLOGIC WORK*

Lillian J. Camagni, A.B., Nutley, N. J.

THE technique of testing bacteriostatic activity in vitro as carried out in this laboratory, and most likely also in other laboratories, involves the repeated flaming of the mouths of the test tubes and of their cotton plugs. It occurred frequently that by flaming, the plugs of neighboring test tubes, and occasionally a whole group of them, were set afire, thus not only causing an undesirable delay in the execution of the experiment, but also increasing the danger of secondary contamination. In order to avoid this disturbing and sometimes even dangerous occurrence, we suggested the use of fireproof cotton as closing wads for the tubes. This proved to be a considerable help in carrying out in vitro tests speedily and with a high degree of safety. We eventually replaced all ordinary cotton wads used for test tubes, flasks, and other containers by fireproof cotton. We have used the fireproof cotton for more than six months; since this modification was found to be most satisfactory, in our laboratory as well as in other laboratories of the research and production depart-

*From the Research Laboratories of Hoffmann-La Roche, Inc., Nutley, New Jersey.
Received for publication, October 13, 1942.

ments of the plant, we feel justified in recommending fireproof cotton for bacteriologic laboratory work.

Fireproof cotton shows no definite difference as compared with ordinary cotton. It stands repeated sterilization in the autoclave as well as in the hot air sterilizer. Temperatures up to 200° C. produce but a very slight browning of the fireproof plugs. Exposure to more than 200° C. or heating at 200° C. for periods longer than two hours should be avoided. The overheating produces a deep brown coloration of the plugs, which are finally disintegrated. They break easily, and a fine brown dust may cover the bottom of the glass containers.

When flamed in the flame of a Bunsen burner, fireproof plugs are only superficially charred, even if exposed for several minutes. There is no danger that the chemicals (e.g., boric acid and sodium tetraborate*) adsorbed in the process of fireproofing might exert a bactericidal or bacteriostatic activity and convey it to the medium if it inadvertently comes in contact with the plug. In order to prove this, the following experiment has been carried out repeatedly:

Sterile wads of fireproof cotton (weight 0.7 to 0.75 Gm.) were drowned in 25 c.c. papain digest broth with 0.2 per cent glucose and kept for twenty-four hours at room temperature (20° C.), in the refrigerator (45° C.), or in the incubator (37° C.). A fourth sample was boiled in the broth for five minutes. The different samples of broth were filled in test tubes, sterilized, and inoculated with typhoid bacilli, hemolytic streptococci, *Streptococcus viridans* and pneumococci Type I. After forty-eight hours' incubation, all tubes showed as normally heavy growth as the controls which had not been in contact with fireproof cotton. The organisms were not altered morphologically and grew typically in subcultures on solid medium.

Fireproof cotton is commercially available† and less expensive than adsorbent cotton. It can, moreover, be prepared in the laboratory.‡

*U. S. Patent 2,185,695.

†We used the fireproof cotton of the Lockport Cotton Batting Company, Lockport, New York.

‡M. Leatherman, 1939, Fireproofing Christmas Trees. U. S. Dept. Agriculture. Leaflet No. 103, 1939.

A SIMPLIFIED SERUM DILUTION METHOD FOR THE QUANTITATIVE TITRATION OF PRECIPITINS IN A PURE ANTIGEN-ANTIBODY SYSTEM*

Donald S. Martin, M.D., Durham, N. C.

INTRODUCTION

The antigen dilution method of precipitin titration, based upon observation of the highest dilution of antigen yielding a visible precipitate in a constant serum-varying antigen series, continues to be used by many investigators, despite the demonstration by Cromwell,[1] in 1925, that an end point obtained by this procedure has no relationship to the antibody content of a serum. This point was emphasized again by Culbertson,[2] who suggested that the antigen dilution method is more applicable to the titration of antigen concentration than to the estimation of antibody content. This procedure is listed as an approved method of precipitin titration in most textbooks of bacteriology, however, and in the latest edition of an authoritative textbook of immunology.[3]

Our interest in the problem arose from a study of the optimal proportions method of precipitin titration, using a photoelectric method for recording the reaction rates of various antigen-antibody mixtures.[4] It was demonstrated that the galvanometer deflection produced by an antigen-antibody precipitate five minutes after mixing was directly proportional to the antigen concentration in the original mixture if there were sufficient antibodies to precipitate all the antigen. The deflection was not changed by any increase in antibody concentration. These observations were in complete agreement with those of Cromwell[1] and Culbertson[2] in that the smallest amount of antigen producing a visible precipitate is a function of the antigen concentration alone, excluding the antigen dilution procedure as a suitable titration method.

On the other hand, the more logical serum dilution procedure has not been used extensively, presumably because of the failure of the earlier workers to obtain precipitates with moderately diluted serum and because it was soon demonstrated that the serum and not the antigen provided the larger part of the precipitate. It seems justifiable to attribute some of the low titers obtained by investigators using the serum dilution method to their use of relatively high concentrations of antigen as the test dose, the inhibiting effects of excess antigen becoming more marked as the relative antigen-antibody ratios are increased in the mixtures containing the higher dilutions of serum.

It was felt, however, that a serum dilution method could be used for the accurate titration of precipitating antibodies if the inhibiting effect of excess antigen could be excluded by using, as a test dose, the smallest amount of antigen yielding a visible precipitate in the presence of excess antibodies.

*From the Departments of Bacteriology and Medicine, Duke University School of Medicine and Duke Hospital, Durham, N. C.

Received for publication, October 22, 1942.

EXPERIMENTAL

The Type I pneumococcic polysaccharide and the refined antipneumococcic rabbit serum (471-11-409-11) were obtained from the Lederle Laboratories through the courtesies of Dr. A. L. Joyner and Miss Frances Clapp.

Sufficient polsaccharide was dissolved in 0.85 per cent saline to make a concentration of 0.512 mg. per c.c. Progressively doubled dilutions of this stock solution were made to give a series of final antigen concentrations ranging from 0.256 mg. per c.c. to 0.0000625 mg. per c.c. after mixing with equal parts of the various serum dilutions. The progressively doubled serum dilutions, prepared in the same way, gave final concentrations ranging from 1:25 to 1:12,800.

The direct precipitin tests were performed by mixing 0.5 c.c. quantities of each reagent in Wassermann tubes. The mixtures were shaken a few times and examined for precipitate formation after standing for two hours at room temperature.

Tests for excess antigen and antibody were made on the supernatants, cleared by centrifugation after the mixtures had stood overnight in the icebox. To one-half of each supernatant was added an equal quantity of a 1:50 dilution of serum, and a solution of antigen containing 0.0085 mg. per c.c. was added to the other half.

The optimal antigen-antibody ratio, obtained by observing the antigen concentration precipitating fastest in a mixture containing a 1:100 dilution of serum, was determined by the photoelectric method.[4]

RESULTS

The results of the direct precipitation tests, presented in the upper half of Table I, are recorded as + or –, no differentiation being made as to the degree of flocculation or turbidity.

The end point in each of the "antigen dilution" titrations was the same (0.00025 mg.), although the most concentrated serum tested (1:25) contained 256 times as many antibodies as the 1:6,400 dilution. These findings were in complete agreement with those of Cromwell[1] and Culbertson,[2] and confirmed our observations obtained with the photronreflectometer.[4] The results are interpreted as eliminating the antigen dilution technique as a quantitative method for determining antibody content.

The "serum dilution" titrations, on the other hand, showed that the end points obtained with the various antigen dilutions were dependent to some extent upon the concentration of antigen used in the test. These end points would not be expected to lie in a straight line because of the well-known fact that precipitation occurs in the region of moderate antigen excess. As the concentration of the test antigen is reduced, however, the end points obtained by the serum dilution technique approach more closely to the true antibody content because of the reduction in the relative amount of excess antigen that can be precipitated by the diluted serum. Consequently it is logical to choose as the test dose of antigen the smallest concentration capable of yielding a visible precipitate with an equivalent amount or an excess of antibodies, in this instance 0.00025 mg. of polysaccharide per c.c. The highest dilution of serum precipitating this amount of antigen must therefore contain just enough antibodies to precipitate this

amount of antigen, there being no excess of precipitable antigen present in the mixture.

If the unit of antibodies be defined as the amount of antibodies equivalent to 1 μg. of antigen, as suggested by Marrack and Smith,[5] this 1:6400 dilution contained 0.25 units giving an antibody titration value of 1,600 units per c.c. of undiluted serum. The same value would have been obtained by using as test antigen the solution containing 0.0005 mg. per c.c. which reacted with the serum diluted 1:3,200. Completely misleading results would have been obtained, however, if a more concentrated test antigen had been used. For example, the antibody value of the serum, based on titration with the antigen solution containing 0.256 mg. per c.c., would have been 25,600 units per c.c., an obviously false value because this dilution of serum did not precipitate all the antigen in the mixture as shown by the presence of excess antigen in the supernatant.

TABLE I

ANTIGEN CONCENTRATION EXPRESSED IN μG. PER C.C.

DIRECT PRECIPITATION TESTS

DILUTION OF SERUM	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
1:25	+	+	+	+	−	+	+	+	+	+	+	−	−
1:50	+	+	+	+	−	+	−	+	+	+	+	−	−
1:100	+	+	+	+	(+)	+	+	+	+	+	+	−	−
1:200	−	+	+	+	+	+	+	+	+	+	+	−	−
1:400	−	−	−	+	+	+	+	+	+	+	+	−	−
1:800	−	−	−	+	−	+	+	+	+	+	+	−	−
1:1600	−	−	−	−	−	+	+	+	+	+	+	−	−
1:3200	−	−	−	−	−	−	−	−	+	+	+	−	−
1:6400	−	−	−	−	−	−	−	−	−	−	+	−	−
1:12800	−	−	−	−	−	−	−	−	−	−	−	−	−
SUPERNATANT EXAMINATIONS													
1:25	A	A	−	a	a	a	a	a	a	a	a		
1:50	A	A	A	−	a	a	a	a	a	a	a		
1:100	A	A	A	A	(−)	a	a	a	a	a	a		
1:200	A	A	A	A	A	−	a	a	a	a	a		
1:400	A	A	A	A	A	A	−	a	a	a	a		
1:800	A	A	A	A	A	A	A	−	−	a	a		
1:1600	A	A	A	A	A	A	A	A	−	−	−		
1:3200	A	A	A	A	A	A	A	A	−	−	−		
1:6400	A	A	A	A	A	A	A	A	A	−	−		

\+ Precipitate
− No precipitate
A Excess antigens in supernatant
a Excess antibodies in supernatant
() Optimal ratio (fastest reacting mixture)

The results of the supernatant examinations are shown in the lower half of Table I. This portion of the table can be interpreted as a series of "neutralization point" determinations, as described by Culbertson,[2] who showed that at this point "antigen and antibody combine in a constant ratio to give the maximum precipitate obtainable from the antiserum." The neutralization method gave very accurate end points except in the highly diluted antigen and antibody mixtures in which supernatant examinations would be expected to fail because the tests for residue antigens and antibodies were made on materials already doubly diluted. As expected, these neutralization points lie in a straight line and calculation from any point in this line shows that 1 c.c. of undiluted

serum contains sufficient antibodies to completely precipitate 1.6 mg. or 1600 μg. of antigen.

With serum diluted 1:50, the fastest precipitation, measured by the photron-reflectometer, occurred when an equal amount of a solution containing 0.032 mg. polysaccharide per c.c. was added. In terms of final concentration, 0.016 mg. of antigen was optimal with 0.01 c.c. of serum, indicating again that this serum contained 1600 units of antibodies per c.c.

In order to emphasize the necessity of using the least amount of precipitable antigen as test material in the serum dilution procedure, the equivalent antigen-antibody ratios, as determined by the neutralization and optimal proportions methods, are connected by a heavy line in the upper portion of Table I.

DISCUSSION

Although the data presented in this paper were obtained with a polysaccharide antigen, there is no reason for doubting its application to the titration of other antigen-antibody systems. Cromwell[1] used sheep hemoglobin and crystalline egg albumin antigens in his experiments, and Culbertson[2] did his work with crystalline egg albumin. At present we are using this method to determine the precipitin response of rabbits to the injection of beef albumin,[6] and in other experiments, we have shown that the galvanometer deflections caused by the precipitation of sheep globulin and albumin are dependent entirely upon the antigen concentration.[7]

All of the methods commonly advocated for the titration of precipitins require the use of a series of mixtures containing constant amounts of undiluted or weakly diluted serum and varying amounts of antigen. In such a constant antibody series the various end points used for estimating the precipitin content can be classified as follows: (A) the highest dilution of antigen yielding a visible precipitate, (B) the dilution of antigen producing the greatest weight or volume of precipitate, (C) the dilution of antigen producing the fastest formation of a precipitate, (D) the highest dilution of antigen completely precipitating the antibodies as indicated by the absence of antigen and antibody in the supernatant, and (E) the determination of antibody N in the precipitate at the equivalence point.

Method A has been shown to have no relationship to the antibody content and need not be considered in spite of its continued use by some observers. Method B yields results which show some relationship to the antibody content, but the method is not quantitative since the greatest weight[8] and the greatest volume[9] of the precipitate are found in the region of antigen excess. Method C is an accurate method of titration if a photoelectric method is used to record the reaction rates in the early phases of the reaction.[4] As originally described by Dean and Webb,[5] the optimal proportions procedure lacked accuracy because these authors suggested that the optimal time for reading the reaction was at the time of beginning particulation. Since the photoelectric method is a specialized procedure requiring the use of an expensive apparatus, it is not recommended for routine use in the titration of pure antigen-antibody systems. This method, however, is the only quantitative method which is applicable to the titration of precipitins in mixtures containing more than one antigen and more than

one antibody. Method D is very satisfactory for the quantitative titration of precipitins in pure antigen-antibody systems but requires centrifugation and special care in selection of the proper dose of antigen for testing the supernatants. Method E is a highly specialized procedure requiring relatively large amounts of serum.[10] The data obtained by this method, however, have been of incalculable value in the advancement of knowledge concerning the chemical factors involved in the precipitin reaction, because the antibody content of a serum can be expressed in terms of actual weight of antibody protein.

The serum dilution procedure, described in this paper, has a distinct advantage in that the titration requires only small amounts of serum; this suggests that this method would be especially valuable in the measurement of residue antibodies in absorbed sera. It must be emphasized, however, that the serum dilution technique, like the microchemical and neutralization procedures, cannot be applied to the titration of complex antigen-antibody systems.

It is unfortunate that the popularity of the antigen dilution technique of titration has resulted in its use by many investigators who often have drawn rather far-reaching conclusions from the data obtained by this method. This point can be illustrated by examination of the data presented in a paper published by Wolfe,[11] which was selected for criticism only because of its recent publication in a leading immunologic journal. This author concluded that chickens were excellent antibody producers, because high precipitin titers could be obtained frequently by a single injection of a small amount of diluted antigen. Although this conclusion may be correct, it is not justified by the data which were obtained by the antigen dilution method. His results show that the sera contained sufficient antibodies to precipitate a minute amount of antigen but give no indication as to the degree of antibody response. The use of the antigen dilution method, however, does not explain the variations in end points obtained by this author, and at least two explanations can be offered for these discrepancies. In the first place, since complex antigens (bovine, sheep, and buffalo serum) were used for immunization and testing, a number of different specific antibodies could have been formed, each antibody being present in a different concentration. A second cause for the variations may be in the use of the rather uncertain "ring test" method of setting up the reactions. The "underlayering" of 0.5 c.c. of an antigen solution with 0.1 c.c. of serum is a rather difficult procedure and introduces the possibilities of error in the measurement of the serum and in obtaining a perfect layer between the antigen and antibody solutions.

The effect of using too concentrated antigen solutions in the serum dilution titration method can be illustrated by a paper published recently by De Gara and Bullowa.[12] These authors, in a study of antibody production in serum sickness, were able to demonstrate precipitins in only one-half of their cases and concluded that serum sickness can occur without the development of observable precipitins. Although this conclusion may be correct, it must be noted that their method was incapable of detecting precipitins unless a very high precipitin titer had been developed by the patient. Since these workers used undiluted foreign sera as antigens, it is obvious that it would take a high concentration of antibodies to overcome the inhibiting effect of these concentrated antigens and to precipitate even a fraction of the precipitable antigen.

SUMMARY AND CONCLUSIONS

A simplified serum dilution procedure is described for the quantitative titration of precipitins in a pure antigen-antibody system.

The titration values obtained by this method are in complete agreement with the values obtained by the "neutralization point" and optimal proportions methods.

The complete lack of relationship between the antibody content of a serum and the titration values obtained by the popular antigen dilution method of titration is discussed.

REFERENCES

1. Cromwell, H. W.: Quantitative Relations Between Antigen and Antibody in the Precipitin Reaction, J. Infect. Dis. 37: 321, 1925.
2. Culbertson, J. T.: A Quantitative Study of the Precipitin Reaction With Special Reference to Crystalline Egg Albumin and Its Antibody, J. Immunol. 23: 439, 1932.
3. Zinsser, H., Enders, J. F., and Fothergill, L. D.: Immunity: Principles and Application in Medicine and Public Health, ed. 5, 1939, Macmillan Company.
4. Martin, D. S.: Use of the Photronreflectometer for Determination of the Optimal Ratio Between Pneumococcus Type I SSS and Antipneumococcus Type I Rabbit Serum, J. Lab. and Clin. Med. 28: 870, 1943.
5. Marrack, J., and Smith, F. C.: Quantitative Aspects of Immunity Reactions: The Precipitin Reaction, British J. Exper. Path. 12: 30, 1931.
6. Martin, D. S., Erickson, J. O., Putnam, F. W., and Neurath, H.: J. Gen. Physiol. In press.
7. Martin, D. S.: To be published.
8. Dean, H. R., and Webb, R. A.: The Influence of Optimal Proportions of Antigen and Antibody in the Serum Precipitation Reaction, J. Path. and Bact. 29: 473, 1926.
9. Dean, H. R.: The Relation Between the Fixation of Complement and the Formation of a Precipitate, Zeitschr. f. Immunitätsforsch. 13: 84, 1912.
10. Heidelberger, M., and Kendall, F. E.: A Quantitative Study of the Precipitin Reaction Between Type III Pneumococcus Polysaccharide and Purified Homologous Antibody, J. Exper. Med. 50: 809, 1929.
11. Wolfe, H. R.: Precipitin-production in Chickens. I. Interfacial Titers as Affected by Quantity of Antigen Injected and Aging of Antisera, J. Immunol. 44: 135, 1942.
12. De Gara, P. F., and Bullowa, J. G. M.: Species-specific Precipitins in Serum Sickness After Intravenous Injection of Refined Concentrated Antipneumococcal Rabbit Serum, J. Immunol. 44: 259, 1942.

AN IMPROVED METHOD OF STERNAL MARROW ASPIRATION*

Joseph Litwins, M.D., New York, N. Y.

THIS communication concerns a description of an improvement in the technique of sternal marrow puncture and aspiration. The improvement consists of the localization of definite landmarks and of the use of a two-way valve either incorporated in the needle or as an adapter attached to the needles now in general use.

The routine technique employed in sternal marrow aspiration consists in puncturing the sternum below the angle of Louis, using the suprasternal notch as a midline landmark. We have found that by placing the middle finger of the free hand on the angle of Louis and the thumb and index fingers on the

*From the Metropolitan and Flower and Fifth Avenue Hospitals, Lbn J. Boyd, M.D., Director of Medicine; Louis Greenwald, M.D., in charge of Hematology.
Aided by a grant from John Wyeth and Brother.
Received for publication, October 22, 1942.

lateral borders of the sternum in the second interspace, unmistakable and immovable landmarks are obtained. The puncture is then made below the middle finger and between the thumb and index fingers. In infants, where the puncture is performed in the manubrium, the middle finger is applied to the suprasternal notch and the thumb and index fingers along the lateral borders of the manubrium.

The sternal puncture needles now in use are superior to the original ones in that they are shorter and permit a firmer grasp in the performance of the procedure; this is of value particularly when the sternal plate is rather hard. In such patients with hard sternal plates, after the marrow is aspirated, it is difficult to remove the needle and syringe simultaneously without at times breaking the tip of the syringe or losing marrow material. It is to prevent such occurrences that this method, the use of a two-way valve, is described. This permits the removal of the syringe having the needle remain in situ until after filling the white cell pipette, and making smears. The needle is then removed.

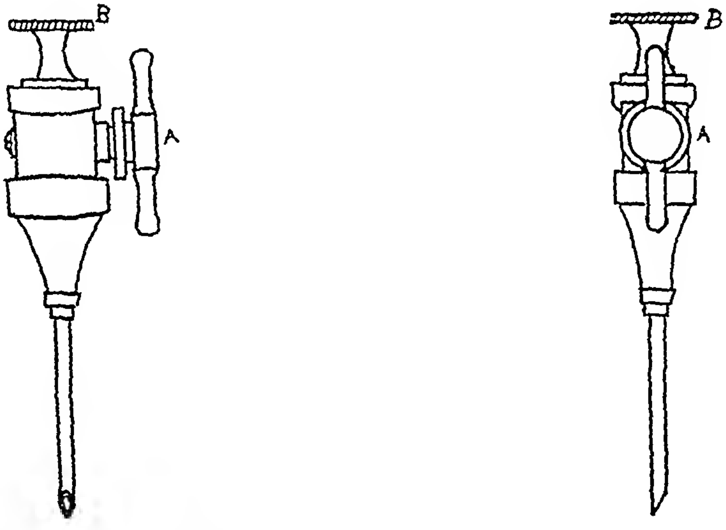


Fig. 1.—Needles used for sternal marrow aspiration. Actual size. *A*, valve control; *B*, stilet.

This may be accomplished by means of a two-way valve either as part of the needle, or as an adapter attachment. By means of the valve one may control the amount of marrow to be aspirated, since often too much marrow is sucked up, causing dilution of the marrow. Again in the so-called "dry" tap, especially in aplastic and osteosclerotic marrow after suctioning and turning off the valve, a small amount of material, sufficient to make one or two smears, may be trapped in the needle.

A three-way valve may be used for sternal transfusions, a procedure which may have to be resorted to rather often in wartime.

AN ORGAN PERFUSION PUMP*

WILLIAM KLEINBERG, ALBERT S. GORDON, AND HARRY A. CHARIPPER,
NEW YORK, N. Y.

AT THE present time increasing importance is being attached to studies dealing with circulatory phenomena in isolated organs. A detailed analysis of such problems necessitates the use of a simple, easily adaptable organ

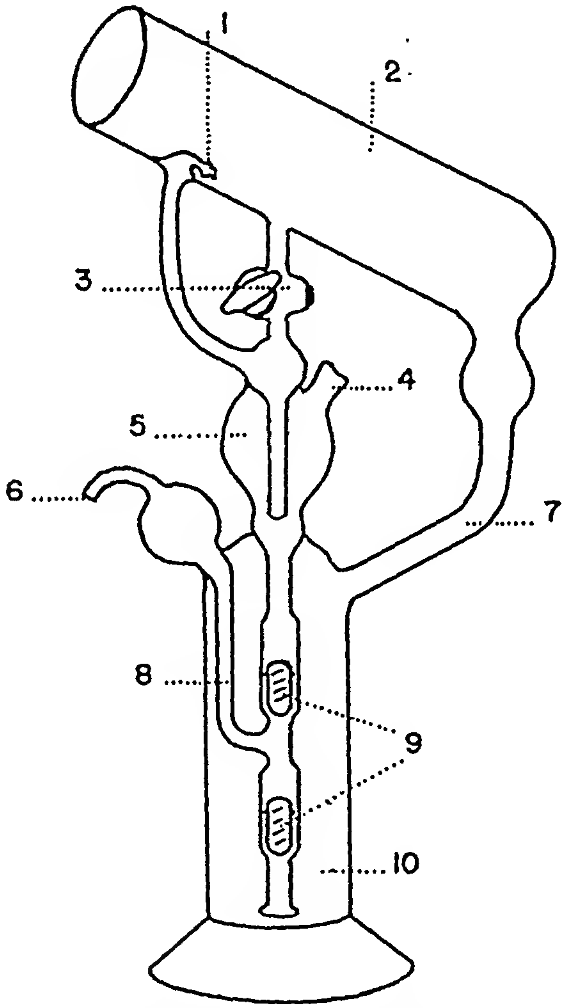


Fig. 1.—(1) cannula, (2) organ chamber, (3) by-pass, (4) outlet to pressure gauge, (5) compression chamber, (6) outlet to rubber bulb, (7) return tube, (8) pulsating fluid column, (9) gravity valves, (10) reservoir.

perfusion pump. The apparatus to be described is a single unit, made of glass and designed for sterile procedures. The mechanism is relatively inexpensive

*From the Department of Biology, Washington Square College of Arts and Science, New York University, New York.

Received for publication, October 27, 1942.

and well adapted for use in small laboratories. All necessary adjustments of systolic, diastolic, and pulse pressure, pulse rate, etc., can be made and changed at will without interrupting the continuous flow.

Perfusion fluid flow is controlled by the action of two ground-glass, gravity valves, under the influence of a pulsating fluid column interposed between the valves. A by-pass stopcock allows any amount of the perfusion fluid and all air bubbles to be shunted and gives precise regulation of pressures. If finer adjustment is desired, the stopcock can be grooved slightly so that the fluid will flow through the grooves when the cock is turned. The necessary elastic properties can be regulated by varying the amount of air in the compression chamber. A pressure gauge is connected to the outlet of this bulb. Filters (glass, silica, platinum mesh, etc.) can be inserted at any point in the return tube or at the bottom of the reservoir.

The rate and amplitude of pulsations are regulated by the rate and degree of compression of a sturdy rubber bulb by any motor mechanism. We have, for example, adapted a sewing machine head to serve this purpose. The systolic pressure is related directly to the extent to which the rubber bulb is compressed. The diastolic pressure may be altered by changing the diameter of the opening in the by-pass. Pulse pressure, therefore, can also be changed at will.

The capacities of the different parts of the pump may be modified to suit individual needs. We have found these to be satisfactory for our work: reservoir, 300 c.c.; organ chamber, 400 c.c.; and compression chamber, 50 c.c.

The blood vessel of the organ to be studied is attached to a cannula and then by a short length of gum rubber tubing to the cannula of the organ chamber. The latter cannula can be filled previously with perfusion fluid by closing the stopcock and applying the drive mechanisms for a few strokes.

The apparatus has been used successfully for studies on perfused endocrine organs, and these will be described in another paper.

THE EFFECT OF FREEZING ON ERYTHROCYTES*

Lloyd Florio, B.A., M.D., Dr. P. H., Mabel Stewart, B.S., Edward R. Mugrage, M.A., M.D., Denver, Colo.

INTRODUCTION

THE statement most commonly made by authorities on hematology is that freezing destroys the human erythrocyte. Since it seemed to us theoretically possible to preserve red blood cells by quick freezing, we were stimulated to study the responses of the erythrocyte to this procedure.

FUNDAMENTAL CONSIDERATIONS

It is possible to freeze and thaw the red blood cell with very little cellular destruction. The speed of freezing and thawing and the concentration of certain sugars added to the blood are fundamental in the preservation of the human erythrocyte by freezing.

TECHNIQUE

Most of the results to be reported were on the human erythrocyte. Blood was drawn aseptically and mixed with dry sodium citrate so that the final concentration of the anticoagulant in the blood was 0.5 per cent. The freezing mixture consisted of ethyl alcohol and solid carbon dioxide which attains a temperature of approximately –75° C. One-half c.c. of blood and 0.5 c.c. of 20 per cent glucose were placed separately into an aluminum cup, 1 mm. in thickness, which is about the size of a water glass. The blood and sugar solution was mixed just before the cup was partially immersed in the cold bath. The best results were obtained if the freezing time was between 3.5 and 5 seconds. We found 1.0 c.c. of mixed blood and glucose the ideal amount of material to work with under the conditions of our experiment. Thawing was accomplished by dipping the cup for 1.5 to 2 seconds in a water bath held at 75° C. In order to study the blood over prolonged periods of time, 1.0 c.c. amounts were frozen, thawed, and pooled. The blood in the pooled specimens was kept refrigerated at 6° C. in sterile flasks. These were allowed to reach room temperature during a one-hour period at the same time each day. The red cells were then counted and the hemoglobin determined. The same pipet and counting chamber were always employed for each sample. Hayem's diluting fluid was used. The hemoglobin was determined by the Sahli technique on the supernatant fluid from a small specimen removed from the flask. The Sahli instrument had been standardized against the Van Slyke oxygen capacity method. In the initial hemoglobin determinations, the supernatant fluid contained so little hemoglobin that the usual Sahli technique could not be used. These initial estimates of hemoglobin were arrived at by color comparisons with samples of the original blood which was hemolyzed to known concentrations of hemoglobin by the addition of distilled water. Fifty milligrams per cent of sulfathiazole was added to both the frozen and control specimens to help

*From the Department of Public Health and Laboratory Diagnosis, University of Colorado School of Medicine, Denver.

Received for publication, October 29, 1942.

maintain sterility although every attempt was made to carry out the entire procedure aseptically. Certain studies carried out by us and several reports in the literature indicate that the addition of this small amount of sulfa drug has no deleterious effect on the blood.

RESULTS ON HUMAN BLOOD

As a result of this procedure 97 to 98 per cent initial preservation was achieved in terms of hemoglobin remaining in the red blood cells and over 99 per cent preservation in terms of cells not destroyed. This may indicate a certain amount of leakage of hemoglobin without destruction of the cell. The hemoglobin concentration in the cell was definitely less than one would expect on the basis of cell count. This is illustrated in Chart I, which represents a typical pooled sample from 20 similar samples of frozen blood followed daily by red cell counts and hemoglobin determinations. The scales on the chart are constructed so that the cell count and the hemoglobin concentration are equivalent.

CHART I

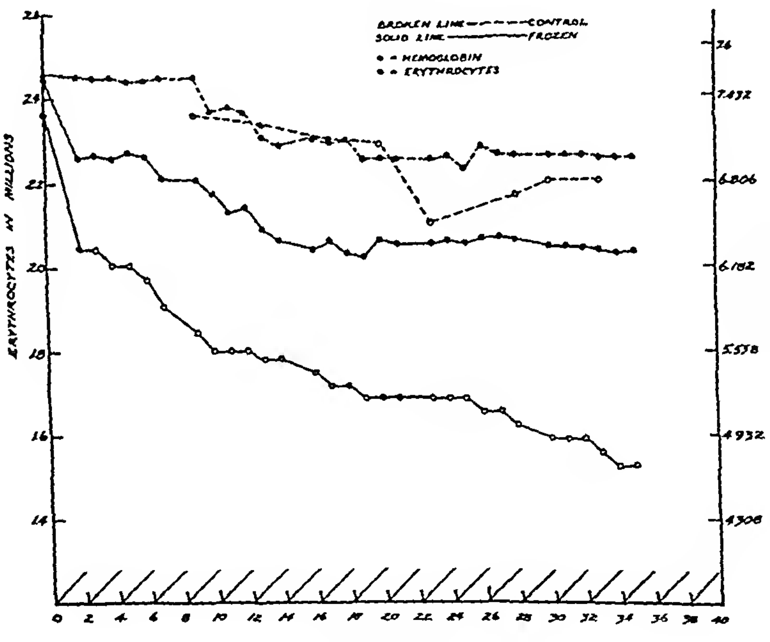


Chart I.—Pooled frozen and control specimens of human blood followed daily by red blood cell counts and hemoglobin determinations.

There was an initial fall in both the cell count and cell hemoglobin in the specimen that was frozen and thawed. After forty-eight hours this leveled off so that a moderately constant relationship was maintained in comparison with the control specimen, which differs only in not having been frozen. Microscopic study always revealed the presence of shadow cells or cells with a much reduced hemoglobin concentration. This was not found in the control.

RESULTS ON OTHER BLOOD

We have made a few studies on the blood of sheep, dogs, and rabbits. The initial results paralleled those found in human blood. The deterioration was

much more rapid, however, especially in respect to hemoglobin leakage, which took place at an accelerated rate even in the control specimen. The accompanying charts illustrate these points.

Unlike the experiments on human blood, however, no attempt has been made to work out the ideal technique for freezing and preservation of the different animal bloods. The conditions found best for the preservation of human red blood cells may not apply to any other blood.

PROPERTIES OF FROZEN BLOOD

Of the many properties of blood we have compared only a few. The oxygen-carrying capacity was unimpaired. Previously frozen cells can be typed with the same ease and accuracy as the unfrozen blood.

Studies on the fragility of the cells revealed peculiarities that depend on the glucose concentration of the specimen. The fragility decreases as the sugar concentration increases, even after the cells were thoroughly washed. Suffice it to say that the fragility of the frozen and unfrozen cells was identical within 0.02 per cent sodium chloride and significantly lower than the same blood not previously mixed with glucose. The whole subject of the fragility of red blood cells suspended in glucose requires further elucidation.

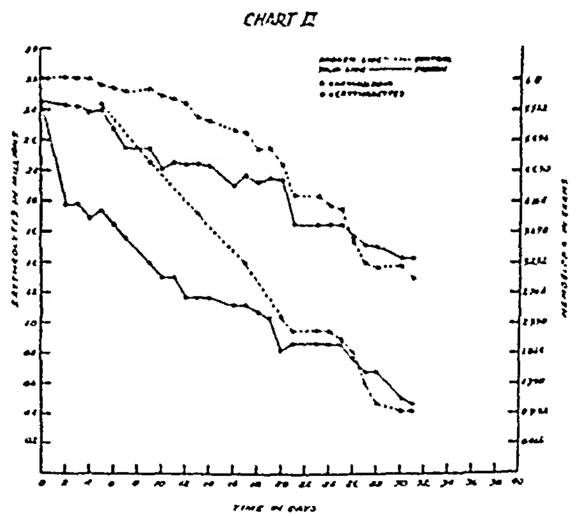


Chart II.—Pooled frozen and control specimens of dog blood followed daily by red blood cell counts and hemoglobin determinations.

Chart III.—Pooled frozen and control specimens of rabbit blood followed daily by red blood cell counts and hemoglobin determinations.

OTHER TECHNIQUES EMPLOYED

Many freezing and thawing techniques were tried but discarded, because the results were not as good as with the procedure which has been described. The blood was sprayed on solid carbon dioxide; small drops were frozen; copper and glass instead of aluminum vessels were used. Blood was also frozen in the ice compartment of an electric refrigerator at –12° C. While there was little hemolysis with this latter procedure if thawed immediately after freezing, destruction of red blood cells took place comparatively rapidly if the blood was kept frozen any length of time.

To thaw, the frozen material was added slowly to fluids of various compositions kept at temperatures ranging between 40° C. and 50° C. or allowed to melt at room temperature. This latter technique always resulted in complete hemolysis except in the case of the blood frozen in the ice compartment.

One might think that freezing of the blood more rapidly, say in one to two seconds, would give better preservation. This was tried but the results were highly unsatisfactory. A mixture of acetone and solid carbon dioxide achieves this quicker freezing. With this mixture, the blood can be frozen in 1.5 seconds. While the temperature of this mixture and our usual one of alcohol and carbon dioxide are the same, the difference in freezing time can probably be explained by the insulating effect of the carbon dioxide bubbles in the alcohol. This phenomenon of bubbling is not observed with the acetone-carbon dioxide mixture. Liquid air was also tried, but it is difficult to handle and the freezing time is about the same as with the alcohol-carbon dioxide mixture.

If we mixed 20 per cent glucose and the blood in equal amounts and allowed this mixture at least twenty-four hours to reach equilibrium before freezing, the results were never as good as when the blood and glucose were mixed within thirty seconds of freezing.

The concentration of plasma does not seem to be a vital factor in preservation. Equally good results were obtained with washed cells resuspended in glucose.

A final concentration of glucose in the blood between 9 and 11 per cent gave good results. More or less sugar resulted in a larger cell destruction on freezing. We routinely employed a 10 per cent final glucose concentration. Galactose, fructose, and sucrose gave less satisfactory results.

While several anticoagulants were quite satisfactory, we consistently used sodium citrate because it is cheap and easy to make up in the desired concentration.

STORAGE AND DRYING IN THE FROZEN STATE

Red blood cells frozen at –75° C. and stored at –12° C. for one hour in the ice compartment of an electric refrigerator invariably are completely hemolyzed on rapid thawing. We have made some attempts to determine the critical temperature at which cells are destroyed. Our experiments suggest that temperatures higher than about –40° C. result in a hemolysis of the erythrocytes. Storage of the blood in a frozen state at the temperature of solid carbon dioxide indicated that there is no deterioration during the first three days of storage. Technical difficulties have made the studies over longer periods inaccurate, but the results to date indicate a slow deterioration in the frozen state from the third to the sixteenth day.

Our few attempts to dry the cells from the frozen state on the cryochem apparatus always resulted in failure. While it is possible to preserve the cell structure when the blood is converted back to a liquid, the cells were practically completely devoid of hemoglobin. They were also extremely fragile and unless care was used in mixing the solid residue and the fluid, even the cell structure was completely destroyed.

SUMMARY

By controlling the freezing and thawing time as well as the concentration of glucose, it was possible to freeze human blood with practically no destruction of the erythrocytes. There seemed to be some leakage of hemoglobin through the cell membrane. This occurred mostly during the first two days following thawing. Pooled specimens of blood that were frozen and thawed deteriorated no more rapidly than the controls after the first forty-eight hours. The same techniques applied to several animal bloods have not been as successful as with human blood. The oxygen-carrying capacity and the ability to type the blood were not altered by freezing. The fragility of the frozen and unfrozen cells was the same. Fragility was decreased in proportion to the per cent of glucose in the blood, however.

Cells frozen at −75° C. were hemolyzed if stored in the ice chamber of an electric refrigerator. We have not found drying from the frozen state successful to date. While it was possible to preserve cell structure, the cells contained practically no hemoglobin.

A STRAIN OF BACTERIUM TYPHOSUM INHIBITED ON BISMUTH SULFITE AGAR*

MARION B. COLEMAN, B.S., ALBANY, N. Y.

THE superiority of bismuth sulfite agar[1, 2, 3] for the isolation of *Bacterium typhosum* and *salmonella* from feces and urine is generally recognized. So much reliance is placed on this medium that the following experience is reported, not to destroy confidence in it, but as an illustration of the importance of using more than one plating medium in any attempt to isolate *Bact. typhosum* from feces.

Cultures of *Bact. typhosum* were isolated from fecal specimens from two patients and a carrier in a limited outbreak of typhoid fever in a rural community in New York state. The microorganism was present in small numbers but was found on bile salts citrate agar media,[4, 5] both before and after enrichment in tetrathionate broth. No typhoid bacilli were isolated from bismuth sulfite agar streaked with the specimens or from that with the enrichment broth. No growth was usually visible with a low power lens (×20) after forty-four hours' incubation of this medium inoculated with pure cultures. When growth occurred, the colonies were generally very small and not typical of typhoid bacilli. Other strains of this species grew characteristically on the same lots of medium.

REFERENCES

1. Wilson, W. J., and Blair, E. M. McV.: A Combination of Bismuth and Sodium Sulphite Affording an Enrichment and Selective Medium for the Typhoid-Paratyphoid Groups of Bacteria, J. Path. & Bact. 29: 310, 1926.

*From Division of Laboratories and Research, New York State Department of Health, Albany.

Received for publication, November 4, 1942.

2. Wilson, W. J., and Blair, E. M. McV.: Further Experience of the Bismuth Sulphite Media in the Isolation of Bacillus Typhosus and B. Paratyphosus B From Faeces, Sewage and Water, J. Hyg. 31: 138, 1931.
3. Wilson, W. J.: Isolation of Bact. Typhosum by Means of Bismuth Sulphite Medium in Water- and Milk-Borne Epidemics, J. Hyg. 38: 507, 1938.
4. Leifson, E.: New Culture Media Based on Sodium Desoxycholate for the Isolation of Intestinal Pathogens and for the Enumeration of Colon Bacilli in Milk and Water, J. Path. & Bact. 40: 581, 1935.
5. Digestive Ferments Company: Bacto SS Agar Dehydrated for the Isolation of Shigella and Salmonella, Difco Laboratories, Inc., Detroit, October, 1940, Circular No. 96A.

AN AUTOMOBILE POLLEN TRAP

JAMES A. MANSMANN, M.D., PITTSBURGH, PA.

THIS is a preliminary report to introduce another simple device and new method for pollen slide exposure, namely, an automobile pollen trap. This trap has been in use since April 1, 1941; daily 24-hour exposures have been made and compared with the results of our many gravity counts. Its counterpart is found in the similar types used in airplane exposures by Stakman,[1] Scheppegrell,[2] and Meier and Lindbergh.[3] C. E. Barrett[4] in 1934 reported a few automobile studies which he had made by exposing slides on the rear seat of his car, but he has not made any contributions since that time.

A DESCRIPTION OF THE TRAP

The trap is a small, oblong, somewhat funnel-shaped box with two open ends and an inner slide shelf. It is principally made up of two parts: the outer part, a covering with the widest end or base attached to the windshield; and the inner part, the shelf upon which the slide rests.

We made a cardboard model first, then placed this design on a thin sheet of aluminum to be cut out with a pair of tin cutters. The trap should be made from lightweight material so that it is not easily detached from the car when meeting strong winds. A thin sheet of aluminum about 20 by 8 inches is ideal and sufficient to make a trap.

The length of the shelf should be such as to allow the 3 inches by 1 inch slide to rest easily upon it. The shelf is a keystone-shaped piece of aluminum with the edges on opposite sides folded one-half inch from the ends at right angles to fasten it to the trap at the proper slant. The entire device is fastened together with lightweight rivets, the ends of which can be turned in with a screwdriver. Wind vents are placed at the base to release the air as it rushes through the trap. Four rubber suction cups are used to attach the trap to the windshield.

Since we have been using a convertible car, the windshield is preferred, but with slight modifications the trap could be placed on the roof or elsewhere just as easily. One precaution should be taken on the windshield attachment. Certain state vehicle codes prohibit any obstructive objects on the windshield,

Received for publication, November 9, 1942.

and in these states a permit must be obtained for application thereon. The importance of where the trap is attached was brought out by Stakman in his airplane studies, when he showed that fewer spores were caught in the George trap when attached to the strut above the surface of the lower wing than when held out of the cockpit by hand.

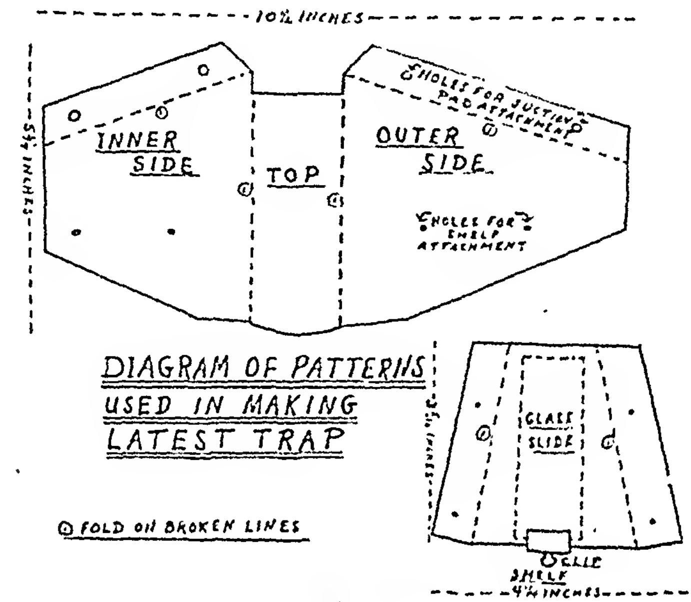


Fig. 1.

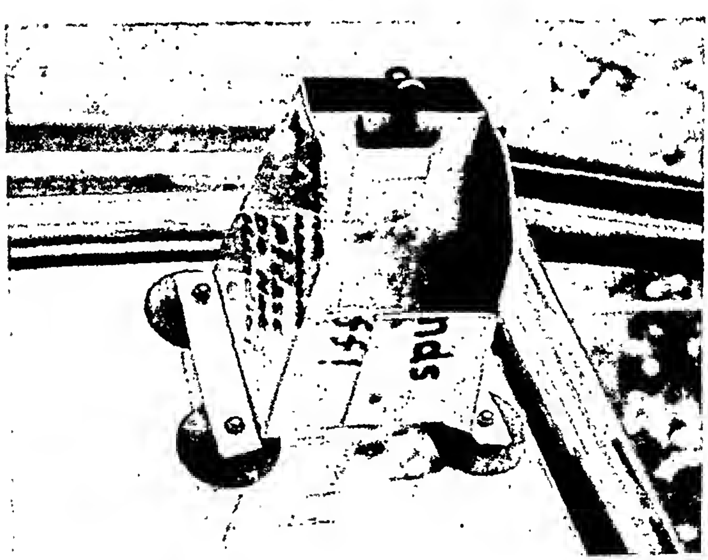


Fig. 2.

The rubber suction cups with a small bolt and nut fastened in the center and a steel clip attached can be purchased at a five-and-ten store. The clips are removed, but can be used to great advantage in anchoring the slide to the shelf. The suction cups allow for easy removal, and the clip makes changing of the slides simple. A one-half inch strip is also turned at right angles at the base on

opposite sides. Two holes are driven through each strip and the suction cups easily bolted on. Wetting the suction surface will give a strong anchorage so that under ordinary driving ranges and meteorologic conditions, the trap will stay in place for about two weeks, after which it should be taken off and re-applied.

Because the windshields vary on different cars, each trap should be fitted to the automobile for which it is to be attached. All the angles are very important, especially the one at which the slide is exposed to the air, and also the one for the protection of the slide from the elements while the car is standing. Of course the design used now favors collection while the car is in motion.

The previous description is a design of a trap which we have used successfully and which we believe with minor changes can be universally adapted to all makes of cars; however, we are constantly experimenting, changing the design and angles to further our knowledge, and hoping for more improvement. We have made many counts with the slide shelf at various angles as it meets the onrushing air, from 30 to 170 degrees. Fig. 3 shows the angle about 75 degrees, but our latest model is placed at 100 degrees. It is our impression that a good deposit of grains is obtained when the slide shelf is placed in a position to meet a great deal of wind resistance.

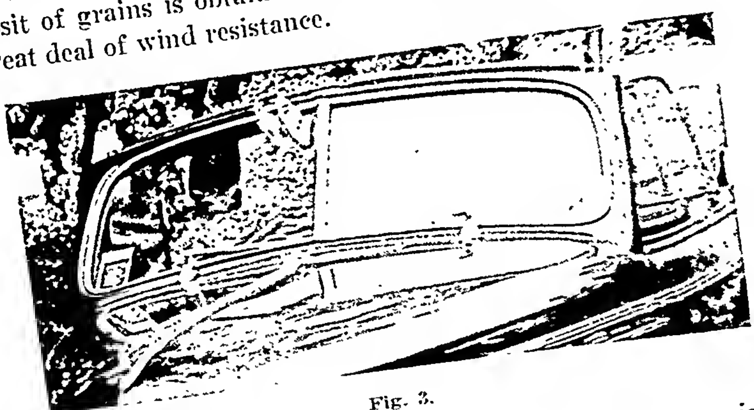


Fig. 3.

Our device has been easy and cheap to construct, a fact we wish to emphasize. It is felt that this trap has the following advantages and with more use can be further developed:

(1) *Simplicity.*—Thus slides for study are easily obtained.

(2) *Mobility.*—The trap can cover any area desired.

(a) This makes pollen studies possible in isolated districts large or small, places in which to look for new types of grains.

(3) *Rapid Sampling.*—This factor will vary with speeds and the exposure angle in the trap. It is from 30 to 150 times faster than the gravity collection.

REFERENCES

1. Stakman, E. C., et al.: Spores in the Upper Air, J. Agricultural Research 24: 599, May, 1923.
2. Scheppegrell, Wm.: Airplane Tests of Hay Fever Pollen Density in the Upper Air—Medical Journal and Record 119: 185, February, 1924.
3. Meier, F. C., and Lindbergh, C. A.: Collecting Microorganisms From the Arctic Atmosphere, Scientific Monthly 40: 5, June, 1935.
4. Barrett, C. E.: Studies in Hay Fever. Clinical Observation Including a Botanical and Air Survey of the Utah Region. J. Allergy 5: 406, 1934.

CHEMICAL

SPECTROPHOTOMETRIC DETERMINATION OF EVANS' BLUE IN SERUM*

O. H. Gaebler, Ph.D., M.D., Detroit, Mich.

The absorption spectra of Evans' blue and oxyhemoglobin are related in such a manner that the dye is readily determined with a spectrophotometer[1] or photoelectric colorimeter[2] in sera showing varying degrees of hemolysis. Constant turbidity introduces no error, but variable turbidity is recognized as a source of uncontrollable errors.[1] In human sera hemolysis is rare if the blood samples are protected with oil, and turbidity is not a common problem. But with dog sera these difficulties are encountered separately or together, particularly in experiments which extend over a period of six hours or more and involve departures from normal conditions.

Results of a preceding study[3] made it possible to choose conditions under which errors due to hemolysis and turbidity can be corrected simultaneously. Correction for turbidity also permits use of 0.8 per cent sodium chloride solution in place of dye-free serum as a blank in spectrophotometric readings. This is advantageous when the dye is given eighteen hours before the experiment, as in "indirect" measurement of changes in plasma volume, and when the dye-free serum is turbid while the dyed sera are clear. In the following procedure one other change in the customary technique is introduced. The sera are diluted with an equal volume of saline solution before reading. This simplifies the calibration and reduces the amount of serum required.

APPARATUS

In the present study a Beckman spectrophotometer[4] equipped with four precision cells of 1 cm. depth was used. Readings in the infrared were required to establish a basis for the simplified technique given below, which is within the range of other instruments.

The cells of the Beckman instrument require 3 c.c. of solution. For admitting to them equal volumes of serum and 0.8 per cent sodium chloride solution the author made a 1.5 c.c. Ostwald-type pipette from capillary tubing having an outside diameter of 7 mm. and a bore of 1.7 mm. The delivery tip of the pipette is 75 mm. long, tapers from 5 mm. to 1 mm. in outer diameter, and has a fine bore. Calibration with water shows that delivery is reproducible to 0.001 c.c.

TECHNIQUE

As in previous methods, paraffin oil is used in the syringes and 15 × 85 mm. specimen tubes to minimize hemolysis. The clotted specimens are centrifuged thoroughly. Attaching a rubber tube to the pipette for suction enables

*From the Department of Laboratories, Henry Ford Hospital.

Received for publication, July 15, 1943.

one to hold the specimens at the level of the eyes, so that serum from small samples can be measured without waste or danger of including cells. While the tip of the pipette passes through the oil, a few bubbles of air should be blown through it. When the pipette has been filled, oil and serum are carefully wiped from the outside and the volume is adjusted.

To 1 volume of each dyed serum one adds 1 volume of 0.8 per cent sodium chloride solution. The extinction (optical density) of the diluted sera is then measured at 750 and 630 mμ. Either dye-free serum diluted in the same manner, or saline solution, may be used as a blank.

CALIBRATIONS

Place 1 volume of the same dye-free serum into each of two cells. To the first cell add 1 volume of saline solution and mix. To the second cell add 1 volume of solution containing 12 mg. of Evans' blue and 8 Gm. of sodium chloride per liter. Using the first cell as a blank, measure the extinction at 750 and 630 mμ. To obtain the coefficients used in calculating Evans' blue concentrations in mg. per liter of undiluted serum, divide the extinction readings by 12. The dilution is ignored, for dyed sera are diluted with saline to the same extent that the saline dye solution is diluted with serum.

As a stock solution of the dye, the one recommended by Gibson and Evans is used. With freshly distilled water and 0.3 Gm. of the dye one prepares 100 c.c. of solution. This is filtered through a sintered glass filter tube, sealed up in ampoules, and autoclaved. The same solution is used for intravenous injection and for preparing the calibrating solution.

The oxyhemoglobin calibration is carried out in the same way, by substituting a saline solution of oxyhemoglobin for the dye solution. The exact concentration of oxyhemoglobin is immaterial, for only the ratio between optical densities at 750 and 630 mμ is needed. The oxyhemoglobin solution mentioned in Table I was prepared by diluting 2 c.c. of citrated blood of a normal dog to 100 c.c. with distilled water, adding 0.8 Gm. of sodium chloride after laking, then filtering through Whatman No. 42 paper.

The turbidity correction is best determined with sera of the type to be encountered in the contemplated study. After diluting the sera with equal volumes of saline, which is also used as the blank, one measures the extinction at 750 and 630 mμ and calculates the ratio between the two values. Somewhat more detailed data required in the present study are shown in Table II. The extinction of eight turbid unhemolyzed dog sera was determined as stated at five or more points between 1000 and 600 mμ. The logarithms of the extinctions were plotted against the negative logarithms of the corresponding wave lengths, and the resulting straight lines[3] were extrapolated to 577 mμ, where traces of oxyhemoglobin interfere with direct measurement of turbidity. From these lines the extinction values shown in Table II were taken. The sera are arranged in decreasing order of turbidity as measured at 630 mμ.

PRINCIPLES OF CALCULATION

The following simultaneous equations present the general problem of determining the concentrations x, y, and z of three substances by spectrophotometry.

$$a_1x + b_1y + c_1z = E_1 \qquad (1)$$
$$a_2x + b_2y + c_2z = E_2 \qquad (2)$$
$$a_3x + b_3y + c_3z = E_3 \qquad (3)$$

One measures the extinction, E_1, E_2, and E_3 of a solution of the three substances at three suitably selected points in the spectrum. The coefficients a, b, and c are determined by calibrating with each substance separately at each of the three points selected. In the present instance E_1 is measured at 750 mμ, E_2 at 630 mμ, and E_3 at 577 mμ. Let y equal the concentration of Evans' blue in mg. per liter of undiluted serum, while x and z represent the oxyhemoglobin concentration and turbidity in any desired units. By calibration one finds that the absorption of the dye at 750 mμ is negligible if the instrument excludes stray light and a concentration of 12 mg. of dye per liter is not greatly exceeded. Thus b_1 is for our purposes zero. Furthermore, one of the reasons for choosing the wave length of 750 mμ is that this makes the ratios a_2/a_1 and c_2/c_1 nearly the same. If these ratios were equal and were represented by k, equations 1 and 2 would yield the following solution for y when b_1 is zero.

$$y = \frac{E_2 - kE_1}{b_2} \qquad (4)$$

TABLE I

EXTINCTION OF OXYHEMOGLOBIN IN DOG SERUM

CELL NO.	SERUM C.C.	OXYHEMOGLOBIN SOLUTION C.C.	SODIUM CHLORIDE 0.8% C.C.	EXTINCTION, 1 CM. CELLS				RATIOS OF EXTINCTIONS		
				750	630	577	540	750	630	577
1	1.5	0.0	1.5	blank						
2	1.5	0.5	1.0	.008	.013	.492	.471	.163	.261	10.0
3	1.5	1.0	0.5	.011	.020	.983	.943	.112	.203	10.0
4	1.5	1.5	0.0	.017	.029	1.50	1.43	.113	.193	10.0

TABLE II

EFFECT OF TURBIDITY AT DIFFERENT WAVE LENGTHS

SERUM NO.	EXTINCTION, 1 CM. CELLS			RATIO OF EXTINCTIONS		
	750	630	577	750	630	577
4	.350	.530	.660	1	1.51	1.89
5	.315	.500	.630	1	1.59	2.00
1	.330	.490	.610	1	1.48	1.85
6	.225	.348	.435	1	1.55	1.93
2	.215	.335	.420	1	1.56	1.95
3	.145	.227	.290	1	1.57	2.00
7	.138	.225	.285	1	1.63	2.07
8	.113	.181	.245	1	1.60	2.17
Average ratios				1	1.56	1.98

In the present case the relationship between the coefficients in equation 3 is such that this simplified solution is sufficiently correct. To demonstrate this, we substitute actual calibration values for the coefficients of x, y, and z in equations 1, 2, and 3. For a_1, a_2, and a_3 we substitute the last set of ratios in Table I. For c_1, c_2, and c_3 we substitute the average ratios at the foot of

Table II. For b_1, b_2, and b_3 we enter values found by calibrating with Evans' blue in the manner described, at 750, 630, and 577 mμ.

$$.113\ x + .0000\ y + 1.00\ z = E_1 \qquad (5)$$
$$.193\ x + .0389\ y + 1.56\ z = E_2 \qquad (6)$$
$$10.0\ x + .0280\ y + 1.98\ z = E_3 \qquad (7)$$

Equation 5

$$z = E_1 - .113\ x \qquad (8)$$

Substitute (8) in (6) and (7)

$$.017\ x + .0389\ y = E_2 - 1.56\ E_1 \qquad (9)$$
$$9.776\ x + .0280\ y = E_3 - 1.98\ E_1 \qquad (10)$$

It is then necessary to divide equation 10 by 575 to obtain the same coefficient for x as in equation 9, and as a result the coefficients of other terms in equation 10 become almost negligible. The final solution is:

$$.03885\ y = E_2 - 1.5634\ E_1 - .00174\ E_3 \qquad (11)$$
$$y = \frac{E_2 - 1.5634\ E_1}{.03885} - .045\ E_3 \qquad (12)$$

Equation 12 differs from equation 4 principally in the presence of an additional term. But if E_3, the extinction at 577, were 2.0, corresponding to 1 per cent transmittance, y, which represents mg. per liter of Evans' blue in serum, would only be changed by 0.09, a matter of less than 1 per cent if the value of y is 10 or over. The value of c_2/c_1 in equations 5 and 6 is 1.56 and that of b_2 is 0.0389. In equation 12 these values have only been altered a few parts per thousand. Whether one should use equation 4, or carry out all calibrations at three points and derive equation 12, is purely a question of whether the two procedures yield as nearly identical results when other types of apparatus and different experimental conditions are employed.

SOURCES OF ERROR

The least complicated determinations, namely, the calibrations with Evans' blue, may be considered first. The same amount of the same serum is present in the blank and the sample under observation; hence, differences in hemolysis and turbidity should not exist. Extinction readings at 750 mμ or longer wave lengths show that this cannot always be taken for granted, for differences in the manner of pipetting or mixing may produce differences in turbidity. If the reading at 750 mμ is zero, the term kE_1 drops out of equation 4. The exactness with which the extinction at 630 mμ, E_2, can be measured, and the constancy of the calibration factor b_2, remain as the limiting factors in accuracy. It is known that extinction measurements between 0.300 and 0.700 are very reproducible with a number of instruments. In the present method the readings will be near the midpoint of this range if the undiluted serum or the calibrating solution contain 12 mg. of dye per liter. This concentration is therefore ideal, and concentrations of 6 to 18 mg. per liter are satisfactory.

The constancy of b_2 was studied in some detail. Four sera were obtained in the course of a week from each of four normal dogs receiving the same diet. Three concentrations of the dye were determined in each of the 16 sera. The data in Table III thus correspond to 48 recoveries of added dye, reported in terms of the observed extinction or optical density. Dividing the values in each line by the concentration of dye indicated in the first column would yield

TABLE III

EXTINCTION OF EVANS' BLUE IN VARIOUS SERA AT DIFFERENT CONCENTRATIONS

MG./L OF DYE IN SOLUTION ADDED TO SERUM	EXTINCTION AT 630 mμ. IN 1 CM. CELLS, AFTER ADDITION TO AN EQUAL VOLUME OF SERUM								MEAN	S.D.
	DOG 1	DOG 2	DOG 3	DOG 4	DOG 1	DOG 2	DOG 3	DOG 4		
3	.121	.106	.117	.117	.111	.107	.108	.115	.113	.005
6	.239	.223	.237	.238	.232	.231	.233	.228	.233	.005
12	.468	.459	.463	.478	.469	.471	.470	.462	.4675	.006
3	.119	.118	.119	.118	.115	.119	.119	.118	.118	.0001
6	.237	.237	.238	.234	.236	.238	.238	.232	.2355	.003
12	.468	.471	.474	.464	.456	.476	.467	.452	.466	.008
Mean of four determinations with 12 mg./L in each animal					.465	.469	.469	.465		
Mean of 16 determinations with 12 mg./L in 4 animals									.467	.007

E_2. With the largest concentration of dye employed the mean result was nearly the same for each animal as for the entire series, and the standard deviation of the series is only 1.5 per cent of the mean. In these 16 determinations the technique was precisely as described above for calibration with the dye.

With smaller concentrations two procedures were used. The data in the first two lines of Table III were obtained by using the technique as described, with the single change that the calibrating solutions contained 3 and 6 mg. of dye per liter instead of 12 mg., the sodium chloride content being constant. In the second set of determinations, only the solution containing 12 mg. of dye per liter was added to serum. The extinction having been read, 1.5 c.c. of the sample was transferred to the next cell and mixed with 1.5 c.c. of a diluent consisting of equal parts of the same serum and saline. The extinction was read, and dilution by the same method was repeated once more. Repeated diluting and transferring caused a very slight increase in turbidity, detected at 750 $m\mu$, for which corrections of .003 or less were subtracted. In contrast with the first set, results with the lower concentrations of dye were quite constant, as indicated by the standard deviation. In either set the extinction was very nearly halved when the highest concentration was halved. In a study in which one uses a number of animals under similar conditions it is therefore satisfactory as well as convenient to use an average of four or more calibrations with representative sera of the group and with one reproducible calibrating solution. When pure solutions of the dye were examined, the extinction at the same concentration and wave length was about 6 per cent greater than in serum, and the specific extinction was perfectly constant for different concentrations. If an instrument does not give the latter result with pure solutions, calibrations with various concentrations of the dye of course become necessary.

The accuracy of the correction for hemolysis and turbidity remains to be considered. This is a question of the constancy of k in equation 4. Considering hemolysis first, we find the necessary data in Table I. Whether the extinction values at 750 $m\mu$ are multiplied by 1.5 or 2.0, the error in predicting the values at 630 $m\mu$ does not exceed .005. E_1 is so small that variations in k are unimportant, provided that one uses concentrations of the dye that give a relatively large value for E_2.

When one dilutes the filtered oxyhemoglobin solution prepared as directed for calibration, the ratio of extinctions at 574 and 620 mμ, or 540 and 620 mμ, which is reported as 40:1 in the literature,[1, 2] progressively falls. This may be due in part to the difficulty of avoiding turbidity completely. In any event it introduces no error in the present method. An oxyhemoglobin solution which was obviously turbid gave a value of only 15.2 : 1 for the above ratio, but the ratio between extinctions at 750 and 630 mμ was near the average of those in Table I.

A serious and incompletely understood source of error related to hemolysis in sera from actual experiments is that a hemolyzed dyed serum not infrequently gives a high value, quite out of line with those for preceding and following unhemolyzed dyed sera. This is true whether the present or previous methods of correction for hemolysis are used. The maximum absorption of Evans' blue is near that of methemoglobin and sulfhemoglobin, and conversion of even a few per cent of the oxyhemoglobin in hemolyzed serum to these derivatives would introduce a large error. The difficulty is probably not due to technical errors and may not be due to the above derivatives, but possible contact of the specimens with traces of oxidizing agents or hydrogen sulfide must be excluded.

TABLE IV

VALUES OF k FOUND BY MEASURING THE EXTINCTION AT 750 mμ (E_1) AND 630 mμ (E_2) IN SERA VARYING FROM EXTREME TO MINIMAL TURBIDITY

E_1	E_2	k	kE_1	E_2-kE_1	E_1	E_2	k	kE_1	E_2-kE_1
.347	.530	1.53	.531	−.001	.030	.058	2.00	.060	−.002
.340	.529		.520	+.009	.029	.060		.058	+.002
.322	.484		.493	−.009	.029	.051		.058	−.007
.254	.404	1.60	.406	−.002	.027	.046		.054	−.008
.251	.398		.402	−.004	.026	.049		.052	−.003
.244	.370		.390	−.020	.024	.043		.048	−.005
.220	.348		.352	−.004	.020	.041		.040	+.001
.201	.325		.322	+.003	.019	.039		.038	+.001
.144	.233		.230	+.003	.019	.039		.038	+.001
.135	.218		.216	+.002	.016	.034		.032	+.002
.100	.171	1.75	.175	−.004	.015	.030		.030	.000
.075	.137		.131	+.006	.015	.030		.030	.000
.058	.105		.102	+.003	.014	.028		.028	.000
.058	.100		.102	−.002	.011	.025		.022	+.003
.045	.080		.079	+.001	.011	.026		.022	+.004
.035	.060		.061	−.001	.010	.020		.020	.000
					.008	.020		.016	+.004
Mean error				.0047					.0025

With reference to turbidity, two situations may be considered. If the control serum and the dyed sera are clear or nearly so, the diluted control serum may be used as a blank. The extinction at 750 mμ will then often be negligible, but will serve as an objective check. Most of the sera in the right half of Table IV could have been subjectively classified as excellent, yet they differ considerably.

If marked turbidity is present in several sera, or in the control serum, the author prefers to make all readings with saline solution as the blank. The value of k in equation 4 was therefore studied in detail, and the results are given in Table IV. Thirty-three sera from normal dogs were diluted with equal volumes of saline and the extinctions were measured at 750 and 630 mμ with

saline solution as the blank. Postprandial as well as postabsorptive sera were included. All sera were of course dye-free. The 16 sera in the left half of Table IV and some of the clear ones in the right half came from specimens drawn and delivered under oil and contained very little oxyhemoglobin. The sera are arranged in descending order of turbidity and are divided into classes with upper limits of 0.030, 0.100, and 0.300 for the extinction at 750 mμ, or E_1. The value of k which most nearly predicts E_2 within these classes is shown, and the mean of the errors in prediction, regardless of sign, is also given. By inspection of the table, it will be noted that an error of .009, or approximately twice the mean error for the turbid sera, is only exceeded in 1 out of 33 sera. This error of .009 corresponds to 0.23 mg. of Evans' blue per liter of serum in the present method, or about 2 per cent if the ideal concentration of 12 mg. per liter is employed.

Evans' blue is removed quantitatively from pure solutions by precipitating calcium oxalate therein. The presence of protein largely prevents this, but the use of serum, as recommended in the original studies on this subject, should be continued. Optical cells used repeatedly with dyed sera presently show extinction values as large as 0.020 at 620 mμ, when filled with saline after thorough rinsing, and should therefore be cleaned with chromic acid or a detergent between determinations. The extinction of the dye at 615 to 630 mμ is not notably altered by changes in pH from 3.0 to 8.0, but stronger alkali changes the color. Sodium hydrosulfite destroys the color in alkaline and weakly acid solutions, an intermediate red stage being noted in the latter.

SUMMARY

Technique for spectrophotometric determination of Evans' blue (T-1824) in serum, with a single correction for both turbidity and hemolysis, is described, and sources of error are examined. When the concentration of dye is 12 mg. per liter and no correction for turbidity or hemolysis is needed, the probable error of individual determinations is 1 per cent. When the correction for hemolysis and turbidity is large, a total error of 4 per cent is possible. To exclude it an adequate number of observations must be made under uniform experimental conditions. Badly hemolyzed dyed sera not infrequently yield unreliable results suggesting the presence of methemoglobin. Any technical errors that could result in formation of this compound or sulfhemoglobin must be avoided. Advantages of the dye over those previously used are well-known; its principal disadvantage is the readiness with which it is adsorbed on glass, calcium oxalate, and protein.

REFERENCES

1. Gibson, J. G., Jr., and Evans, W. A., Jr.: Clinical Studies of the Blood Volume. I. Clinical Application of a Method Employing the Azo Dye "Evans' Blue" and the Spectrophotometer, J. Clin. Investigation 16: 301, 1937.
2. Gibson, J. G., Jr., and Evelyn, K. A.: Clinical Studies of the Blood Volume. IV. Adaptation of the Method to the Photoelectric Microcolorimeter, J. Clin. Investigation 17: 153, 1938.
3. Gaebler, O. H.: The Relationship of Extinction to Wave-length in Turbid Sera and Other Suspensions, J. Biol. Chem. 149: 251, 1943.
4. Cary, H. H., and Beckman, A. O.: *A Quartz Photoelectric Spectrophotometer*, J. Optic. Soc. America 31: 682, 1941.

DENSITY FINDINGS WITH HEPARIN IN BLOOD, PLASMA, AND SERUM*

Alfred L. Copley, M.D.,† and Mary E. Whitney, Kansas City, Kan.

WHILE studying viscosities of heparinized blood we[1] found a decrease in the density with heparin. This led us to investigate further the influence of heparin added in various units to blood, plasma, and serum.

The densities were determined by pyknometry, and calculated by the equation, $\text{Density} = \frac{\text{Weight}}{\text{Volume}}$. Two Nicol tube pyknometers with capacities of 1.2517 c.c. and 2.2869 c.c. were used. The blood was obtained in clean dry 50 c.c. syringes. Adequate accuracy to measure 0.3 c.c. quantities of liquid in question was provided by the use of precision Pyrex pipettes, Mohr Measuring Type, with capacities of 0.1 and 0.2 c.c. It was found essential that the glassware employed be rigorously cleaned.

Blood was taken from three human beings and diluted 9:1 with anticoagulant solutions. Not less than 9 c.c. of blood were used for each dilution. Two, 5, 25, and 125 units of heparin (110 Murray-Best units per milligram of the sodium salt) were contained per c.c. of these blood systems. For controls, 0.2 per cent sodium oxalate, 0.2 per cent potassium oxalate, and 0.3 per cent sodium citrate in equal volumes of blood dilution were used. One portion of each of the blood systems was centrifuged at 3,000 revolutions per minute for twenty minutes, and the supernatant plasma was tested for density. All tests were run continuously within six hours following blood withdrawal. With increasing amounts of heparin, the density decreases in both blood and plasma, but not in linear proportion. Samples containing heparin show lower density than the controls. Results are compiled in Table I. Similar results, as with sodium oxalate, were obtained with potassium oxalate and sodium citrate.

Serum from two human beings, three dogs, and one sheep was obtained six hours following blood withdrawal by centrifugation at 3,000 revolutions per minute for twenty minutes. Three-tenths c.c. solutions of 10, 50, 250, and 1,250 units of heparin in distilled water and, as control, 0.3 c.c. distilled water were added to 3 c.c. serum, and pyknometry was done within three hours. In all instances the density was lowered with heparin. The decreases were more marked with higher heparin concentration, although they were never directly proportional (Table II).

Since Chargaff and Olson[2] found that protamine deionizes and flocculates heparin, we have determined the influence of various amounts of heparin on the density of a 1.5 per cent protamine sulfate‡ solution in physiologic saline. Following centrifugation of the protamine solution at 1,500 revolutions per

*From the Hixon Laboratory for Medical Research, University of Kansas, Kansas City.

†University of Virginia Hospital, Charlottesville, Va.

‡A sample of protamine sulfate was kindly supplied by E. R. Squibb & Sons, New Brunswick, N. J.

Received for publication, April 12, 1943.

minute for ten minutes, heparin solutions were added to the decanted fluid. In each case the density was decreased by heparin.

TABLE I

IN VITRO EFFECT OF VARIOUS AMOUNTS OF HEPARIN UPON DENSITY OF HUMAN BLOOD AND PLASMA AT 27° C.

CASE	FLUID	PER C.C. OF BLOOD-ANTICOAGULANT MIXTURE 9:1				
		UNITS OF HEPARIN				SODIUM OXALATE
		2	5	25	125	0.2 PER CENT
A	Blood	1.0589	1.0588	1.0586	1.0584	1.0593
	Plasma	1.0290	1.0288	1.0284	1.0279	1.0291
B	Blood	1.0549	1.0546	1.0547	1.0540	1.0558
	Plasma	1.0241	1.0240	1.0238	1.0234	1.0242
C	Blood	1.0488	1.0489	1.0168		1.0495
	Plasma	1.0227	1.0224	1.0219		1.0233

TABLE II

INFLUENCE OF VARIOUS AMOUNTS OF HEPARIN UPON DENSITY OF SERUM OF DIFFERENT SPECIES

SERUM	TEMPERATURE °C.	UNITS OF HEPARIN IN 0.3 C.C. DISTILLED WATER ADDED TO 3 C.C. SERUM				
		0	10	50	250	1250
Human I	26	1.0232	1.0230	1.0229	1.0228	1.0224
Human II	27	1.0247	1.0238	1.0237	1.0235	1.0231
Dog I	27	1.0274	1.0269	1.0269	1.0261	1.0255
Dog II	27	1.0263	1.0261	1.0260	1.0259	1.0257
Dog III	29	1.0191	1.0189	1.0187	1.0185	1.0183
Sheep	29	1.0233		1.0224	1.0223	

SUMMARY

Heparin has a specific lowering action upon the density of the four systems of blood, plasma, serum, and protamine sulfate solution. Increasing amounts of heparin decrease the density more markedly, but not in linear proportion. Data are not, as yet, sufficiently extensive to draw any conclusions as to the mechanism of this action.

REFERENCES

1. Copley, A. L., Krchma, L. C., and Whitney, M. E.: Humoral Rheology. I. Viscosity Studies and Anomalous Flow Properties of Human Blood Systems With Heparin and Other Anticoagulants, J. Gen. Physiol. 26: 49, 1942.
2. Chargaff, E., and Olson, K. B.: Studies on the Chemistry of Blood Coagulation. VI. Studies on the Action of Heparin and Other Anticoagulants: The Influence of Protamine on the Anticoagulant Effect In Vivo, J. Biol. Chem. 122: 153, 1937.

VI. A SIMPLE AND ACCURATE METHOD FOR QUANTITATIVE DETERMINATION OF BILE ACIDS AND SALTS IN BILE

Lester M. Morrison, M.D., Philadelphia, Pa.

In 1940, in this journal, the author, in collaboration with W. A. Swalm, gave a brief, preliminary description of a simple, stalagmometric method for the quantitative determination of bile salts in urine and bile.[1] During the intervening years, this procedure has been utilized in the author's laboratory as one of the routine laboratory aids for the estimation of hepatic function in diseases involving the liver or biliary tract.[2,3] This experience in application of the procedure in the analysis of samples of bile obtained by intubation of the duodenum has indicated some possible sources of error and the need of amplifying the original description of the technique.

APPARATUS

Fig. 1 illustrates the stalagmometer, which may be obtained from the Precision Thermometer and Instrument Co., of Philadelphia, Pa. It consists of a pipette to which is attached a capillary tube of 7.5 cm. in length and 0.106 cm. in diameter. Graduations A and B on the pipette define a volume of 13.2 c.c. Changes in surface tension are measured in terms of numbers of drops falling from the capillary outlet as the fluid contained in the pipette flows from graduations B to A.

In order to prevent plugging of the capillary tip with precipitated particles, the stalagmometer should be cleaned each day at the beginning and at the end of a series of analyses by drawing cleaning solution as high as the safety bulb above graduation B. After draining off the cleaning solution, first water is drawn into the apparatus in a similar manner and then an alcohol-ether mixture. If, in this process of cleaning, water enters the needle valve, this should be dried first by an alcohol-ether mixture and then by a current of air. Finally, the stalagmometer is dried by a current of air.

CALIBRATION OF THE STALAGMOMETER

For a given volume of water or bile, different stalagmometers may deliver varying numbers of drops. Hence, each stalagmometer should be calibrated in order to determine corrections to be applied in calculation of results. For this purpose, a stock solution is prepared containing 100 mg. of pure cholic acid in 100 c.c. of 0.01 NaOH. From this stock solution, serial dilutions are made up by adding each of the following aliquots, viz., 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 c.c. water in 100 c.c. volumetric flasks. These solutions are diluted to approximately 98 c.c. To each of these solutions, 6 N hydrochloric acid is added drop by drop with agitation until one drop, transferred by means of a stirring rod to a strip of filter paper saturated with tropeolin 00, shows a change from yellow to pink. After acidification, all solutions are diluted to 100 c.c.

Calibration of the stalagmometer should include first determinations of numbers of drops of distilled water falling from the stalagmometer during the

Received for publication, April 16, 1943.

flow of the water from the upper to the lower graduation. By means of the needle valve, the rate of fall of the drops must be adjusted to 30 per minute. Then readings are made of each of the diluted and acidified solutions of cholic acid prepared as described above.

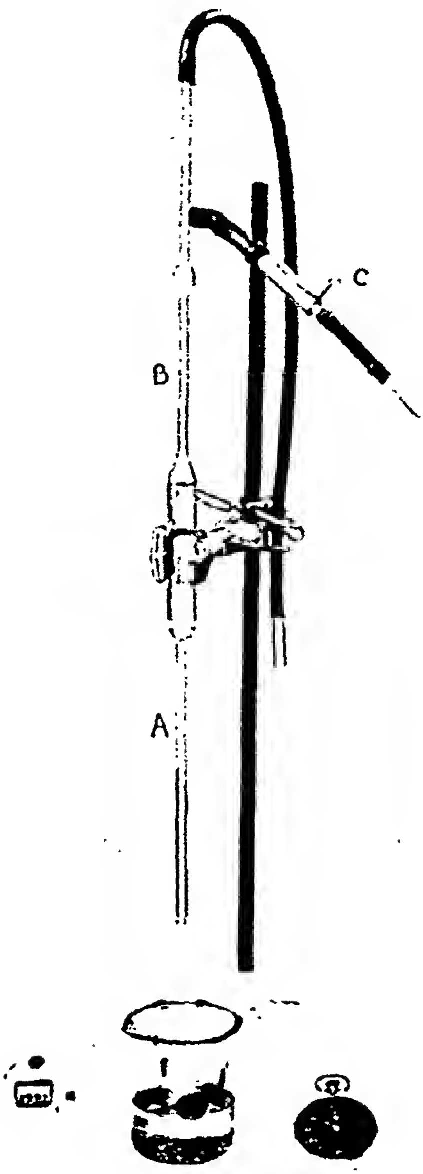


Fig. 1.

Concentrations of cholic acid in water corresponding to varying numbers of drops from one stalagmometer are shown in Table I. A similar table may be prepared for the particular stalagmometer being calibrated, or the readings may be corrected to conform with those in Table I. The procedure for determining corrections for a particular stalagmometer, A, is illustrated by the following example.

Before numbers of drops from stalagmometer A are referred to Table I for calculations of results, the numbers of drops must be multiplied by 0.94.

ANALYSES OF SPECIMENS OF BILE OR BILE FRACTIONS

Chart I shows the relationship between decrease in surface tension of water, as determined by the stalagmometer, and the concentration of cholic acid in solution. The maximum rate of rise of the curve lies within the range of concentrations from 2.0 to 5.2 mg. of cholic acid per 100 c.c. corresponding to stalagmometric readings from 157 to 190 drops. Within these levels, changes in concentration of cholic acid have greater effects upon the numbers of drops falling from the stalagmometer than do similar changes in other parts of the curve. In other words, this range represents the sensitive part of the curve. All readings should be maintained within the limits of 157 to 190 drops, as reported in Table I.

CHART I

CONCENTRATION OF CHOLIC ACID IN WATER MG. PER 100 C.C.	NUMBER OF DROPS FROM A	NUMBER OF DROPS TABLE I	RATIO DROPS—TABLE I / DROPS—FROM A
0	156	144	0.92
2.0	166	157	0.94
4.0	192	183	0.95
6.0	204	192	0.94
	Average	=	0.94

An inspection of the bile fraction will suggest the proper range of dilution. Fluid specimens are diluted 10 or 25 times and viscid samples 50 to 100 or even 200 times.

A measured amount of bile (0.5 to 2.5 c.c.) is diluted with water to about 24 or 49 c.c. in a 25 or 50 c.c. glass stoppered volumetric cylinder. It is acidified with 6 N hydrochloric acid to the end point of the tropeolin 00 test paper using an outside drop test, and it is made up to the 25 or 50 c.c. volume mark.

TABLE I

TABLE FOR CALCULATION OF CONCENTRATIONS OF CHOLIC ACID FROM STALAGMOMETRIC READINGS

NUMBER OF DROPS	0	1	2	3	4	5	6	7	8	9
140					0	0.50	0.80	1.00	1.20	1.30
150	1.40	1.50	1.60	1.70	1.80	1.90	1.95	2.00	2.05	2.10
160	2.15	2.20	2.25	2.30	2.35	2.40	2.45	2.50	2.55	2.60
170	2.70	2.80	2.90	3.00	3.10	3.20	3.30	3.40	3.50	3.60
180	3.70	3.80	3.90	4.00	4.10	4.30	4.40	4.60	4.80	5.00
190	5.20	5.50	5.80	6.20	6.60	6.90	7.30	7.60	8.00	8.30

Values in the table give mg. per 100 c.c. of cholic acid.

The stalagmometer is filled by aspiration and allowed to drip into a 10 or 25 c.c. cylinder. The flow of drops is adjusted to 30 drops per minute or less. The drops are counted in the usual manner, but a reading is taken when the solution in the cylinder reaches a level of an even c.c. mark. Another reading is taken when the solution reaches the next c.c. level. The difference in readings represents the number of drops in 1 c.c. of solution. A reading can be taken on the next c.c. as a check. A previous calculation from the desired range

of readings (160 to 190 drops) and the volume of the stalagmometer (13.2 c.c.) will indicate when the number of drops per c.c. of the solution will give a total reading within the desired range.

$$\frac{160}{13.2} \text{ to } \frac{190}{13.2} \text{ is 12 to 14.4 drops per c.c.}$$

If the number of drops per c.c. is below the desired range, a fresh solution of higher concentration is prepared.

If the number of drops per c.c. is above the desired range, the solution is diluted 1:1 with adjustment of the pH to the specified end point. If the reading indicated more than a 1:1 dilution, it is advisable to prepare a fresh solution.

The stalagmometer is rinsed and filled with the new solution and the counting begun. If the preliminary test shows the reading is within the desired range, the stalagmometer reading of the full volume is taken.

As the solution in the stalagmometer flows out, the rate of flow diminishes to 20 drops a minute. It can be readjusted to 30 drops by a manipulation of the needle valve without affecting the reading.

CALCULATION OF RESULTS

Stalagmometric readings for 2 or more dilutions of the bile, giving numbers of drops within a range of 160 to 190, are referred to a table prepared for that particular instrument; or readings corrected for the instrument, as described above, are referred to Table I. The concentration of cholic acid listed in the table corresponding to the reading is then multiplied by the dilution of the bile.

For example, a sample of bile diluted 1:100 gives, in duplicate determinations, 185 and 187 drops. The mean of 186 drops in Table I indicates a concentration of 4.4 mg. per 100 c.c. Hence, the concentration of bile salts as cholic acid in the bile fraction is 4.4 × 100 = 440 mg. per 100 c.c.

TABLE II

SUMMARY OF RECOVERIES OF CHOLIC ACID ADDED AS DESOXYCHOLIC ACID, CHOLIC ACID, OR BILE SALTS TO FRACTIONS OF BILE DRAINED FROM HUMAN SUBJECTS

SOURCE OF CHOLIC ACID ADDED TO BILE	RANGE OF CONCENTRATION OF CHOLIC ACID IN BILE MG. PER 100 C.C.	CHOLIC ACID ADDED MG. PER 100 C.C.	NO. OF EXPERIMENTS	AVERAGE RECOVERY PER CENT OF ADDED CHOLIC ACID	AVERAGE DEVIATION
Cholic Acid	103 – 138	25 – 140	7	104	±5
	360 – 480	75 – 500	6	99	±9
	615 – 990	240 – 960	9	108	±4
Desoxycholic Acid	300 – 390	38 – 380	12	96	±11
	570 – 700	60 – 600	7	97	±6
Bile Salts	103 – 107	47 – 100	3	104	±3
	300 – 480	42 – 500	13	98	±5
	615 – 990	32 – 960	18	100	±4
	Averages		75	103	±7.3

PROBABLE ERROR OF THE METHOD

The accuracy of the method has been determined by a series of 75 recovery experiments summarized in Table II. The cholic acid concentrations of samples

of bile drained from human subjects were reinforced by the addition of either pure cholic acid, pure desoxycholic acid, or a commercial (Fairchild's) preparation of bile salts. Analyses of several samples of this commercial preparation of bile salts yielded an average of 34 per cent of cholic acid.

Quantities of cholic acid added to the bile samples gave theoretical increases varying from approximately 10 to 100 per cent above the original levels determined in these samples. Average recoveries of added cholic acid for the different levels of cholic acid concentration in the samples of bile varied from 96 to 108 per cent. For the 75 analyses, the average recovery was 103 per cent with an average deviation of ± 7 per cent.

SUMMARY

This report summarizes experiences over a period of three years with a simple, stalagometric method for determination of bile acid and salts in samples of bile drained by intubation of the duodenum.

In the description of the technique, particular emphasis has been placed upon conditions of dilution and acidification of samples of bile, and the range of stalagmometric readings which have yielded most satisfactory results.

A series of 75 experiments have given an average recovery of 103 ± 7.3 per cent of cholic acid, added either as cholic acid, desoxycholic acid, or a mixture of bile salts to samples of bile drained from human subjects.

REFERENCES

1. Morrison, L. M., and Swalm, W. A.: I. Studies of Liver Function in Health and Disease. Observations on a Simple and Accurate Method for Quantitative Determination of the Bile Salts in Urine and Bile, J. Lab. & Clin. Med. 25: 739, 1940.
2. Morrison, L. M.: IV. Studies of Liver Function in Health and Disease. A New Test of Liver Function Determined From the Concentration of Bile Salts in Bile and Urine, Am. J. Digest. Dis. 7: 527, 1940.
3. Morrison, L. M., and Swalm, W. A.: Preliminary Report of a New Physiologic Test for Liver Function, Rev. Gastroenterol. 7: 269, 1940.

THE SEPARATE DETERMINATION OF THE FATTY ACID FRACTION AND OF THE NEUTRAL FAT PLUS STEROL FRACTION IN FECES*

J. C. Forbes, Ph.D., and T. T. Atkinson, Jr., B.S., Richmond, Va.

The separate determination of fatty acids and of the neutral fat portion in feces theoretically offers valuable information as to the efficiency of fat digestion and absorption. Analytical procedures have been described by a number of investigators, e.g., Tidwell and Holt,[1] Fowweather,[2] Holt, Courtney, and Fales,[3] and Saxon,[4] but all the procedures are quite long and require considerable manipulation. Several years ago a relatively simple method for the determination of neutral fat and cholesterol in animal tissue was described by Outhouse and Forbes.[5] Since a simple method for the determination of fat and free fatty acids should be of considerable value in clinical chemical lab-

*From the Department of Biochemistry, Medical College of Virginia.
Received for publication, May 3, 1943.

oratories, it was decided to determine whether this method could be modified in order to allow the independent determination of the neutral fat fraction and fatty acids in a mixture of the two. After attempting various procedures, a method was finally worked out and applied with satisfactory results to fecal analyses. No distinction is made between fatty acids present as soaps or as free fatty acids. Sterols are included in the neutral fat fraction. Bloor's oxidative technique[6] with minor modifications was used throughout.

Determination of Neutral Fat Plus Sterols.—Approximately 3 Gm. of feces are air-dried, as recommended by Tidwell and Holt,[1] ground thoroughly, and last traces of moisture removed in a desiccator. About 0.3 Gm., accurately weighed, is ground in a mortar with 5 Gm. of doucil,* an artificial zeolite, and transferred quantitatively to a 125 c.c. glass-stoppered Pyrex bottle. 100 c.c. of chloroform are added, followed by 0.8 c.c. of 10 per cent sodium hydroxide to precipitate the free fatty acids as their sodium soaps. The bottle is shaken well and set aside with occasional shaking for several hours and preferably left overnight. The chloroform solution is filtered through a fat-free rapid filter paper, and 2 to 5 c.c., depending upon the apparent amount of fat present in the sample, are transferred to a 125 c.c. glass-stoppered Pyrex Erlenmeyer flask. The flask is placed in warm water, and the solvent is completely removed with the aid of a stream of air played upon the surface of the liquid. Five c.c. of a silver chromate solution in sulfuric acid are added, and this is followed by 3 c.c. of 1 N potassium dichromate. The flask is stoppered loosely, the solution gently mixed, and the flask put in an electric oven at 95° C. The flasks are removed after about five minutes, rotated to stir up the contents, stoppered tightly, and replaced in the oven for two hours. Control flasks using the same amount of silver chromate and potassium dichromate solutions are run at the same time. The stoppers are removed immediately after the flasks are taken from the oven, and shortly thereafter approximately 60 c.c. of cold distilled water are added. Ten c.c. of 10 per cent potassium iodide are added without stirring, and the liberated iodine is titrated without delay with 0.1 N sodium thiosulfate, using 1 per cent starch solution as indicator. A small granule of sodium hydroxide should be added to the potassium iodide solution to prevent decomposition. The starch solution should not be added until the yellow color of the iodine has practically disappeared. The difference between the titration value of the control and unknown solution is a measure of the lipids present in the unknown sample taken for analysis. A value of 3.6 c.c. of 0.1 N sodium thiosulfate is taken as equivalent to 1 mg. of neutral fat. Sterols are extracted along with the neutral fat and appear in the calculations as neutral fat. Phospholipids do not interfere as they are removed by the doucil.

SPECIAL REAGENTS REQUIRED

Silver Chromate Reagent.—To 5 Gm. of silver nitrate dissolved in 25 c.c. of water are added 5 Gm. of potassium dichromate dissolved in 50 c.c. of water. The precipitated silver dichromate is separated by centrifugation, washed twice with water by centrifugation, and then dissolved without drying in 500 c.c. of pure concentrated sulfuric acid.

*The doucil was obtained from W. A. Taylor and Company, Baltimore, Maryland.

Sodium Thiosulfate Standard (0.1 N) Solution.—Weigh out 25 Gm. of ordinary C.P. sodium thiosulfate or 24.83 Gm. of the pure dry recrystallized salt. Dissolve in water and dilute to a liter. Boiled distilled water must be used. This solution is standardized against acid potassium iodate. Weigh accurately 0.3249 Gm. of acid potassium iodate. Dissolve in 50 c.c. of water, heating gently if necessary. Transfer the solution to a 100 c.c. flask, rinsing the beaker carefully, and dilute to volume with water. This solution is exactly decinormal. Pipette 25 c.c. into an Erlenmeyer flask, add 1 gram of potassium iodide dissolved in a little water and a few cubic centimeters of dilute hydrochloric acid. Titrate immediately with the thiosulfate solution, using starch as indicator.

Determination of Soaps Plus Free Fatty Acids.—A weighed amount, 0.1 to 0.2 Gm., of the thoroughly ground sample is transferred to a 125 c.c. Pyrex glass-stoppered bottle and 4 c.c. of 1:4 sulfuric acid added (1 vol. of sulfuric acid plus 3 vols. of water). The bottle is warmed gently on a hot plate so as to convert all soaps to free fatty acids. This heating should be continued until the solution is close to the boiling point and considerable water has condensed on the sides. The flask is allowed to cool and 5 Gm. of doncil added. After mixing, 100 c.c. of chloroform are added, and the bottle is shaken thoroughly, then is set aside with occasional shaking for several hours, and preferably is left overnight. The chloroform solution is filtered through a fat-free rapid filter paper, 2 to 5 c.c. of the filtrate are evaporated to dryness in an oxidation flask, and the total lipid content is determined by the oxidation method already described. Since the oxidation value of the fatty acids approximates that of the corresponding fat, 3.6 c.c. of 0.1 N sodium thiosulfate has been taken as equivalent to 1 mg. of lipid material, whether present as neutral fat or fatty acids. The difference between the lipid content per 100 Gm. as determined above and that obtained for the neutral fat portion gives the fatty acids present as soaps or as free fatty acids.

TABLE I

RECOVERY OF NEUTRAL FAT AND FREE FATTY ACIDS FROM MIXED SOLUTIONS

EXPERIMENT NUMBER	LIPIDS PRESENT			LIPIDS RECOVERED				
	TOTAL GM.	NEUTRAL FAT GM.	FATTY ACID GM.	TOTAL GM.	NEUTRAL FAT OBTAINED GM.	NEUTRAL FAT CORRECTED GM.	FATTY ACID OBTAINED GM.	FATTY ACID CORRECTED GM.
1	.1391	.0577	.0814	.1358	.0614	.0594	.0744	.0764
2	.1451	.0445	.1006	.1363	.0470	.0450	.0893	.0913
3	.0955	.0417	.0538	.0952	.0409	.0389	.0543	.0563
4	.1086	.0502	.0584	.1068	.0520	.0500	.0548	.0568
5	.1086	.0502	.0584	.1075	.0544	.0524	.0531	.0551
6	.1829	.1056	.0773	.1812	.1130	.1110	.0682	.0702
7	.1531	.0773	.0758	.1481	.0812	.0792	.0669	.0689
8	.1147	.0630	.0517	.1140	.0622	.0642	.0518	.0538
9	.2109	.0902	.1207	.2092	.0992	.0972	.1100	.1128
Total	1.2585	.5804	.6781	1.2341	.6113	.5973	.6228	.6408
			Per cent error	−1.1	+5.3	+2.9	−8.1	−5.5

Before applying the method to fecal analyses, it was necessary to determine the recovery from known solutions of fat and fatty acid. In these experiments a weighed amount of recrystallized tripalmitin and stearic acid was dissolved in 200 c.c. of chloroform. Approximately half of the solution was transferred to a 125 c.c. glass-stoppered bottle containing 5 Gm. of doncil, after which

0.8 c.c. of 10 per cent sodium hydroxide was added and the solution thoroughly shaken. The remainder was transferred to a similar bottle containing 5 Gm. of doneil plus 4 c.c. of 1:4 sulfuric acid. Both bottles were thoroughly shaken and allowed to stand with occasional shaking for several hours, and usually left overnight. They were then filtered and analyzed as previously described, and the amount present in the original 200 c.c. of solution was calculated. In the calculations 3.60 c.c. of 0.1 N sodium thiosulfate have been taken as equivalent to 1 mg. of tripalmitin and 3.66 c.c. for 1 mg. of stearic acid. The experimental results are shown in Table I. The values for neutral fat are on the average approximately 5 per cent too high. This is due primarily to a slight solution of the fatty acids in the chloroform, even in the alkaline medium. Experiments with pure stearic acid under the condition used showed an average solubility of approximately 1 mg. per 100 c.c. of chloroform. If corrections are made for this solubility, the per cent recovery is quite satisfactory. In the usual fecal analysis an error of this magnitude can be neglected. This has been done in obtaining the experimental results recorded in Table II.

TABLE II

LIPID ANALYSIS OF NORMAL FECES

EXPERIMENT NUMBER	NEUTRAL FAT PLUS STEROLS	FATTY ACIDS PLUS SOAPS
	Per cent	Per cent
1	8.3	10.9
2	6.5	8.2
3	10.9	2.9
4	7.5	9.3
5	6.7	10.9
6	8.7	7.1
7	8.0	8.9

All values are given in per cent dry weight.

Experiments with calcium stearate following the procedure described for soaps and free fatty acids gave satisfactory recovery of the stearic acid. The analytical results obtained on normal feces and recorded in Table II are in good agreement with the commonly accepted normal values. Although approximately 0.3 Gm. is very suitable for normal feces, the sample should be reduced to about half this value if the fat content is apparently high.

SUMMARY

A relatively simple method for the determination in feces of neutral fat plus sterols, free fatty acids, and soaps is described.

REFERENCES

1. Tidwell, H. C., and Holt, L. E.: The Estimation of the Total Lipids and the Lipid Partition in Feces, J. Biol. Chem. 112: 605, 1935.
2. Fowweather, F. S.: The Determination of the Amount and the Composition of the Fat of Feces, Brit. J. Exper. Path. 7: 7, 1926.
3. Holt, L. E., Courtney, A. M., and Fales, H. S.: A Method for the Determination of Fat in Dried Feces and Its Distribution as Soap, Free Fatty Acids and Neutral Fat, Am. J. Dis. Child. 17: 38, 1919.
4. Saxon, G. J.: A Method for the Determination of the Total Fats of Undried Feces and Other Moist Masses, J. Biol. Chem. 17: 99, 1914.
5. Outhouse, E. L., and Forbes, J. C.: A Micromethod for the Determination of Tissue Lipids, J. Lab. & Clin. Med. 25: 1157, 1940.
6. Bloor, W. R.: The Determination of Small Amounts of Lipids in Blood Plasma, J. Biol. Chem. 77: 53, 1928.

A SIMPLE AND ACCURATE METHOD FOR THE DETERMINATION OF MERCURY IN BIOLOGIC MATERIAL*

THOMAS H. MAREN, A.B., NEW BRUNSWICK, N. J.

THE use of dithizone as a colorimetric reagent for mercury is due to Fischer and Leopoldi,[1] and Fischer,[2] and has recently been adapted to use in biologic materials by Laug and Nelson.[3]

The colorimetric method is accurate enough to supplant tedious older methods, which depend upon electrolysis or amalgamation of the metallic element for its ultimate determination, or upon titration methods involving iodide or thiocyanate. The present method has the advantage of extreme simplicity, speed, and a broad range. A single analysis should not take longer than five minutes, after digestion of organic material is completed.

DISCUSSION

The investigators cited above have regarded copper as an interfering element. They separated copper from mercury at pH 1 by the use of KI, KBr, or $Na_2S_2O_3$. Finally, the mercury dithizonate is formed at pH 6. Both of these steps are eliminated in the present method. The number of operations in the procedure are thereby considerably reduced. Formation of the mercury dithizonate at pH 1 further eliminates the problem of maintaining lead free reagents and apparatus. At pH 6 dithizone is extremely sensitive to lead, and in the earlier methods special consideration of this was required.

We have found that copper need not interfere, if the proper conditions of pH are maintained. In acid solution, dithizone reacts with gold, palladium, platinum, mercury, silver, copper, and bismuth. The latter do not, however, react at pH 0 to 1.0. In 50 c.c. 0.25 N HCl, 1,000 mg. of copper does not change the color of dithizone reagent. Silver does not react in the presence of excess halogens. Mercury and the noble metals alone, therefore, react with dithizone under these conditions, and the latter will rarely interfere with analysis of biologic materials. The yellow color of the dithizone-mercury complex may then be regarded as specific in the presence of 0.25 N HCl and in the absence of the noble metals. The presence of 5 to 7 c.c. sulfuric acid (from the organic digestion) brings the pH to 0.2 and does not alter the above considerations in any way.

Fig. 1 shows that at 490 $m\mu$ conditions are proper for a "mixed color" determination of mercury with a spectrophotometer. Here the green dithizone solution transmits rather freely, and the yellow mercury complex has its absorption maximum. Solutions to be measured will range from green through light green and dark yellow to light yellow. The standard or unknown is measured against the dithizone solution. Beer's law is obeyed; the optical density has a linear relation to the concentration of mercury.

In the digestion of organs and urine, it was not found necessary to connect a water-cooled condenser to the digestion flask. A mixture of equal weights of sulfuric and nitric acids was used. It was found that the digestion proceeds

*From the Wallace Laboratories, Inc.

Received for publication, May 28, 1943.

smoothly if about three times as much mixed acid as organic material is used. Occasional charring may occur; the addition of small supplementary amounts of nitric acid will obviate this. The digestion is carried out in a micro-Kjeldahl flask with ground glass joint. The digestion may proceed with an air-cooled condenser rather than under reflux. We have found that elimination of the water-cooled condenser considerably decreases the time of digestion and no loss of mercury is involved. Table I shows recovery of mercury using the method described below. Table II shows noninterference of copper over a wide range.

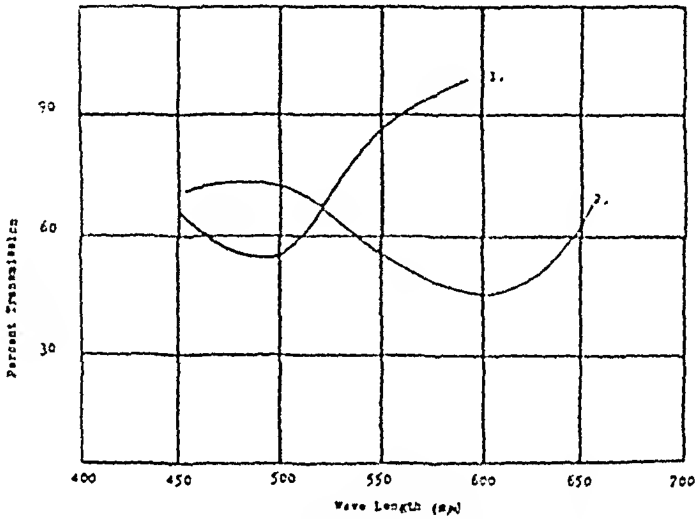


Fig. 1.—1. Mercury-dithizone complex ($CHCl_3$ solution). 2. Dithizone (in $CHCl_3$). (Readings from Coleman spectrophotometer. Solutions shaken with .25 N HCl in all cases.)

REAGENTS

1. Sulfuric Acid. Analytical Reagent.

2. Nitric Acid. Analytical Reagent. An equal weight mixture of 1 and 2 was used as the stock digestion solution.

3. Hydroxylamine hydrochloride chemically pure. A 20 per cent aqueous solution is used to destroy any excess nitric acid.

4. 0.25 N HCl. From chemically pure HCl.

5. Dithizone solution. 10 mg. dithizone (Eastman) dissolved in 1 liter of chloroform. This is a light green solution. (Transmission curve shown in line 2 of Fig. 1.) 10 c.c. will be entirely converted to the metal salt by 25 micrograms of mercury. The resulting yellow solution is plotted in line 1 of Fig. 1.

PROCEDURE

To about 5 Gm. of organic material add 10 to 15 c.c. of acid digestion mixture in a long-necked Kjeldahl flask, to which is fitted (using a glass joint) an air condenser about 18 to 20 inches long. Digestion begins spontaneously. After ten minutes apply a gentle flame, and increase the heat gradually until the full flame of the burner is used. After an hour the nitric acid fumes disappear and the digestion is finished.

The ash solution is cooled and poured into a separatory funnel containing 25 c.c. of .25 N HCl and 2 c.c. of 20 per cent hydroxylamine hydrochloride. Add 10 c.c. of dithizone reagent solution, shake 50 times, and collect the $CHCl_3$

TABLE I

SUBSTANCE	AMOUNT	MICROGRAMS HG ADDED	TYPE OF CONDENSER	MICROGRAMS HG FOUND (RECOVERY)
Rabbit blood	1 c.c.	10	Water-cooled	9.8
Rabbit blood	5 c.c.	10	Water-cooled	9.9
Rabbit blood	1 c.c.	10	Air-cooled	9.8
Rabbit blood	1 c.c.	10	Air-cooled	9.7
Guinea pig liver	4 Gm.	10	Air-cooled	10.0
Guinea pig liver	3 Gm.	5	Air-cooled	4.9
Human urine	20 c.c.	20	Air-cooled	20.2
Human urine	20 c.c.	20	Air-cooled	20.0

TABLE II

SUBSTANCE	AMOUNT	MICROGRAMS HG ADDED	MICROGRAMS CU. ADDED	TYPE OF CONDENSER	MICROGRAMS HG FOUND (RECOVERY)
Rabbit blood	5 c.c.	5	0	Air-cooled	4.9
Rabbit blood	5 c.c.	5	5	Air-cooled	5.0
Rabbit blood	5 c.c.	5	15	Air-cooled	5.0
Human urine	5 c.c.	5	50	Air-cooled	4.8
Human urine	5 c.c.	5	100	Air-cooled	4.9
Rabbit blood	5 c.c.	5	100	Air-cooled	5.1

layer. The mercury dithizone thus formed is very sensitive to light and should not be exposed to bright sunlight. The extract is measured with the spectrophotometer against dithizone at 490 mμ, and the reading is compared with that on a standard curve.

This calibration curve, conforming to Beer's law, sets optical density against concentration of mercury. The standards may be prepared by adding known amounts of mercury (not exceeding 25 mg.) to 25 c.c. of 0.25 N HCl, and extracting, as above, with 10 c.c. dithizone. The optical density of the extract is measured against that of dithizone solution at 490 mμ. We have not found it necessary to include the other reagents used in the digestion in this standardization. They have no effect on the final color. Twenty-five milligrams of mercury will convert all the dithizone to the yellow mercury complex. Larger amounts therefore must be determined by taking aliquot samples of the unknown after digestion.

An alternate procedure, which is accurate enough for general biologic work, may be employed in the absence of a spectrophotometer. In this method the mercury is titrated with dithizone solution. For the concentration of dithizone used, 1 c.c. of the solution is equivalent to 2.5 mg. of mercury. Small portions of dithizone are added to the funnel, which contains, as above, the mercury sample 0.25 N HCl and hydroxylamine hydrochloride. The end point is reached when the dithizone in the funnel is olive green, indicating slight excess of unreacted reagent. The number of micrograms of mercury in the sample is equal to the cubic centimeters of dithizone solution x 2.5. It is advisable, however, to standardize the dithizone solution by actual titration against a known amount of mercury.

The titration method is also useful when over 25 mg. of mercury are present. In this case the spectrophotometer cannot be used, for 10 c.c. of dithizone reagent is completely converted to the mercury salt, and a "mixed color" determination is impossible. Successive additions of dithizone, however, will yield the green and point color. The total amount of dithizone added will then be a measure of the mercury present.

SUMMARY

A rapid, sensitive, colorimetric method for analysis of mercury, particularly in biologic materials, has been presented. Dithizone in $CHCl_3$ reacts with mercury at pH 1 (only gold palladium and platinum would interfere) to form a yellow complex which may be analyzed by the "*mixed color method*" in the spectrophotometer, or may be used as the basis for direct titration of the mercury.

The author wishes to thank Miss Blanche Kohut for her technical assistance.

REFERENCES

1. Fischer, H., and Leopoldi, G.: Quantitative Bestimmung kleiner Quecksilbermengen mit Dithizone, Z. Anal. Chem. 103: 241, 1935.
2. Fischer, H.: Dithizonverfahren in der Chemischen Analyse, Angew. Chem. 50: 919, 1937.
3. Laug, E. P., and Nelson, K. W.: Report on Mercury, J. Assoc. Official Ag. Chem. 25: 399, 1942.

DETERMINATION OF DIODRAST-IODINE IN URINE AND IN FILTRATES OF PLASMA*

A. C. CORCORAN, M.D., AND IRVINE H. PAGE, M.D.
INDIANAPOLIS, IND.

THE accurate determination of diodrast-iodine has become widely practiced in the determination of diodrast clearance and of functioning tubular secretory mass (Smith, Goldring, and Chasis,[1]). The following simple procedure has proved very satisfactory in daily use during the past two years. The method was orginally developed from Alpert's procedure[2] as a titrimetric procedure in which diodrast-iodine was determined as iodate by thiosulfate titration. The colorimetric procedure used by Flox, Pitesky, and Alving[3] was then applied to it without modifying other aspects of our method.†

Principle.—"Duponol PC"‡ is used to stabilize recovery of diodrast from plasma filtrates. Diodrast-iodine is oxidized by treatment with bromine water in acid solution. The excess of bromine is removed by addition of sodium formate, and the iodate iodine (Flox, Pitesky, and Alving,[3] 1942) content is determined photocolorimetrically.

Preparation of Reagents.—1. Acid $ZnSO_4$ with Duponol PC. 27 Gm. $ZnSO_4$ and 30 c.c. of approximately 1 N H_2SO_4 are made up to about 1 liter with water. One gram of Duponol PC is added and put in solution with the least possible shaking. Ten c.c. of the mixture in about threefold dilution are titrated with 0.15 N NaOH, using phenolphthalein as indicator. The pink color of the end point should persist for one minute. From the value obtained in the titration, the acid $ZnSO_4$ solution is diluted until 10 c.c. of it exactly neutralizes 11.2 c.c. of 0.15 N. NaOH.

2. 0.15 N NaOH. The solution is prepared by dilution of "carbonate-free" NaOH in recently boiled distilled water. It is protected from atmospheric CO_2 by a soda-lime trap. Plasma filtrates prepared as directed below should be of pH 6.7 to 6.8. The recovery of diodrast from plasma becomes less complete as

*From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital.
†Details of this procedure were furnished us before its publication through the kindness of Dr. Alving.
‡E. I. Du Pont and Company, Wilmington, Del.
Received for publication, May 31, 1943.

the pH range 6.0 to 6.2 is reached, while the plasma "blank" increases rapidly about pH 6.8. The accuracy of preparation of reagents should therefore be checked by the determination of pH in plasma filtrate before the solutions are placed in use. The addition of Duponol PC increased the pH range over which recovery of diodrast-iodine from plasma is possible and permits full recovery at plasma dilutions of less than 1:15.

3. Bromine water. An excess of liquid bromine is added to 500 c.c. of distilled water. The mixture is stored in the refrigerator, water and bromine being replenished as required.

4. Sodium formate. 10 Gm. of reagent grade sodium formate are dissolved in 100 c.c. of water. The solution is kept in the refrigerator and made freshly each week.

5. Alkaline KI. 50 Gm. of KI, reagent grade, are dissolved in approximately 100 c.c. of 0.01 N NaOH. The solution is protected from light by storage in an amber dropping bottle.

PROCEDURE

1. Precipitation of plasma proteins: A modification of the Somogyi zinc precipitation is used. To one volume of heparinized plasma are added five volumes of acid $ZnSO_4$ with Duponol. The mixture is allowed to stand for about thirty minutes. Five volumes of 0.15 N NaOH are then added dropwise with constant agitation. The mixture is filtered at once through a No. 1 Whatman paper *without* prior centrifugation.

Because of the high dilutions (1:1000) used, comparable precipitation of protein from urine samples is unnecessary except in the presence of gross proteinuria or hematuria. In such cases, 0.5 c.c. each of the $ZnSO_4$ and NaoH solutions are added to the 1 or 2 c.c. samples of urine in a volumetric flask, the mixture then made up to volume with water and a sufficient amount filtered.

2. Oxidation of Diodrast-Iodine to Iodate: 0.2 c.c. of 85 per cent $H3PO_4$ is added to 10 c.c. of plasma filtrate or diluted urine in a colorimeter absorption tube. One c.c. of bromine water is added and the mixture shaken until homogenous. The tube is then placed in a boiling water bath for three minutes, when it is rapidly cooled to room temperature in an ice bath. The wall of the tube is then carefully dried by wiping with a soft towel, and 1 c.c. of 10 per cent sodium formate added. The solutions are mixed by shaking and then allowed to stand 20 to 30 minutes at room temperature.

3. Measurement of Color Density: The clarity of the mixture before addition of KI permits determination of the individual "center-settings" of each tube, thus correcting for inaccuracies in the calibration of individual tubes and permitting the use of tubes which have become too scratched or worn for other uses. The "center-setting" is determined for each tube in the series, using the appropriate color filters. One drop of alkaline KI is then added, the "center-setting" is appropriately adjusted, and the reading is made at exactly two minutes.

Light filter No. 400 is suitable for the determination of plasma "blank" and of plasma diodrast-iodine concentration during clearance periods. Filter No. 440 is ordinarily used for the determination of diodrast-iodine in urine dilutions and No. 490 for the concentrations in plasma and urine which obtain in determination of Tm_D.

Calculation.—The determination of diodrast-iodine concentration from

galvanometer readings is made by reference to a calibration curve from the preparation either of iodate solutions or from dilutions of 35 per cent diodrast solution treated with all reagents exactly as described. The separately measured plasma "blank" should be subtracted from the observed plasma concentration, and the result multiplied by the dilution factor (11); thus (0.175 to 0.002) 11.0 = 1.925 mg. per 100 c.c. of plasma, where 0.0175 is the apparent diodrast-iodine content of the 10 c.c. of unknown plasma filtrate and 0.002 the value of the plasma "blank."

The high dilutions of urine used obviate any appreciable error from urinary "blank."

Results.—The mean recovery in 34 determinations of diodrast-iodine added to human plasma was 100 per cent. The range of recovery in this series varied from 97.5 to 102 per cent. The concentrations used ranged from 1.9 to 66 mg. per 100 c.c. of plasma.

We found the method particularly useful because: (a) There is the convenience and safety of bromine water and sodium formate in contrast, respectively, with bromine and phenol reagents; (b) the separate determination of "center-setting" for each tube obviates errors due to differences between individual tubes, such as result from etching or scratches and permits the use of tubes, which would otherwise be discarded; and (c) the use of 10 c.c. of plasma filtrate in a final volume of 12.2 c.c. yields a color density from 1.0 mg. per 100 c.c. plasma diodrast-iodine which, using filter No. 400, reaches at about the center of the galvanometer. The use of a special filter for determination of low concentrations of iodate-iodine is thus avoided.

The use of "Duponol PC" was practiced because it was found that its detergent action made it possible to obtain full recovery of plasma diodrast in zinc filtrates in which the plasma had been diluted only 10 or 11 times, whereas only 90 to 95 per cent was recovered in these filtrates in the absence of "Duponol PC," when it was added in large excess or when a series of other detergents were tried. Full recovery of diodrast-iodine, however, may be obtained from plasma in zinc filtrates without the use of the detergent when the plasma is 15 times diluted.[3] We have found the more concentrated filtrates advantageous, and therefore retain the use of "Duponol PC." Probably some other application in clinical chemistry of this action of detergents will be found.

SUMMARY

A convenient method is described for determination of diodrast-iodine in urine dilutions or plasma filtrates. The method depends upon the detergent action of "Duponol PC" for full extraction of diodrast from plasma: the diodrast-iodine of the plasma filtrates is then oxidized with bromine water and the excess of bromine decomposed by adding sodium formate. The iodate-iodine content of the samples is determined photocolorimetrically by the method of Flox, Pitesky, and Alving.[3]

REFERENCES

1. Smith, H. W., Goldring, W., and Chasis, H.: The Measurement of the Tubular Excretory Mass, Effective Blood Flow and Filtration Rate in the Normal Human Kidney, J. Clin. Investigation 17: 263, 1938.
2. Alpert, L. K.: Rapid Method for Determination of Diodrast-Iodine in Blood and Urine, Bull. Johns Hopkins Hosp. 68: 522, 1941.
3. Flox, J., Pitesky, I., and Alving, A. S.: A Direct Photoelectric-Colorimetric Method for the Determination of Diodrast and Iodines in Blood and Urine, J. Biol. Chem. 142: 147, 1942.

MEDICAL ILLUSTRATION

COLORING AND APPLYING FACIAL PROSTHESES*

CAPTAIN CARL DAME CLARKE, SANATORY CORPS, ARMY OF THE UNITED STATES
WASHINGTON, D. C.

IN CONSIDERING the color result of a final prosthesis it must be realized that there are seven distinct steps that can be used in color work alone to obtain the finished product. All of these are not necessary in every instance, but each step is worthy of consideration. The procedure begins with the latex mix and does not end until cosmetics are applied to the prosthesis when it is in place on the patient. The steps taken to produce the color effect are as follows:

1. Counteracting the natural yellow color of rubber and its tendency to darken.
2. The addition of water-soluble pigments and dyes to the latex mix to produce a basic monochrome skin color suitable to the individual patient.
3. Blended color effects on the dried rubber prosthesis achieved with oil-soluble dyes.
4. Detail effects produced with alcohol-soluble dyes.
5. Placing of hair, glass eyes, and fingernails in the rubber cast.
6. Highlighting or lacquering the surface.
7. The application of cosmetics.

These steps are of sufficient importance to require a detailed description of each.

1. *Counteracting the Natural Yellow Color of Rubber.*—Unfilled rubber turns dark on prolonged exposure to sunlight. It assumes a yellow cast which varies from light cream to raw umber. Steps to forestall this change must be taken while the rubber is in the latex state. Zinc oxide (a thickening agent) and clay or chemical fillers should be added. The zinc oxide should not exceed 2 per cent in vulcanized latex or 4 per cent in unvulcanized latex; otherwise the final prosthesis may crack. This cracking can occur even when the prosthesis is soft and pliable. An excess of zinc oxide would cause the prosthesis to become so opaque that it would not possess the translucency of normal Caucasian skin.

In addition to the zinc oxide, clay fillers can be added to prevent the rubber from becoming too dark. The clay fillers should not exceed 10 per cent or the translucency will be lost. In fact, if the rubber contains both zinc oxide (2 per cent) and clay filler (10 per cent), the prosthesis may lack the translucency of normal Caucasian skin. The proportions should invariably be less than the percentages mentioned. The mixture is balanced to suit specific types of skins. It is often better to make up stock solutions of the rubber

*From the Department of Moulage and Prosthetics, Army Medical Museum.
Received for publication, July 9, 1943.

latex containing different percentages of clay filler. I believe the clay fillers are more suitable than zinc oxide for this purpose. If clay is used various stock solutions of latex can be prepared, each having the proper basic colors incorporated, and each being slightly different from the other. A dried sample from each lot may be attached to the neck of the bottle. These samples are placed next to the patient's skin to determine which solution is most suitable

Fig. 1.—The front view of a patient requiring a prosthesis.

for making the final prosthesis. If zinc oxide is used instead of clay to prevent darkening, the stock solution may in time coagulate in the bottle and become unfit for further use. When zinc oxide is chosen, it must be incorporated as a thin water paste into the latex just before it is poured into the mold. Stock solutions containing a small amount of zinc oxide that are kept for long periods of time become so thick that it is difficult to pour them into

molds having small openings. If the stock solution is diluted with water, it continues to sink in the mold because the plaster absorbs the water, and air pockets will likely form in the cast. These may be corrected by refilling the mold as the solution sinks in the opening. Some stock solutions containing zinc oxide thicken considerably in twenty-four hours. Although this may be an advantage when it is necessary to build up a coagulum deposit, as on agar molds of hands, it is a disadvantage in molds that have small openings as in a prosthesis of the ear or nose.

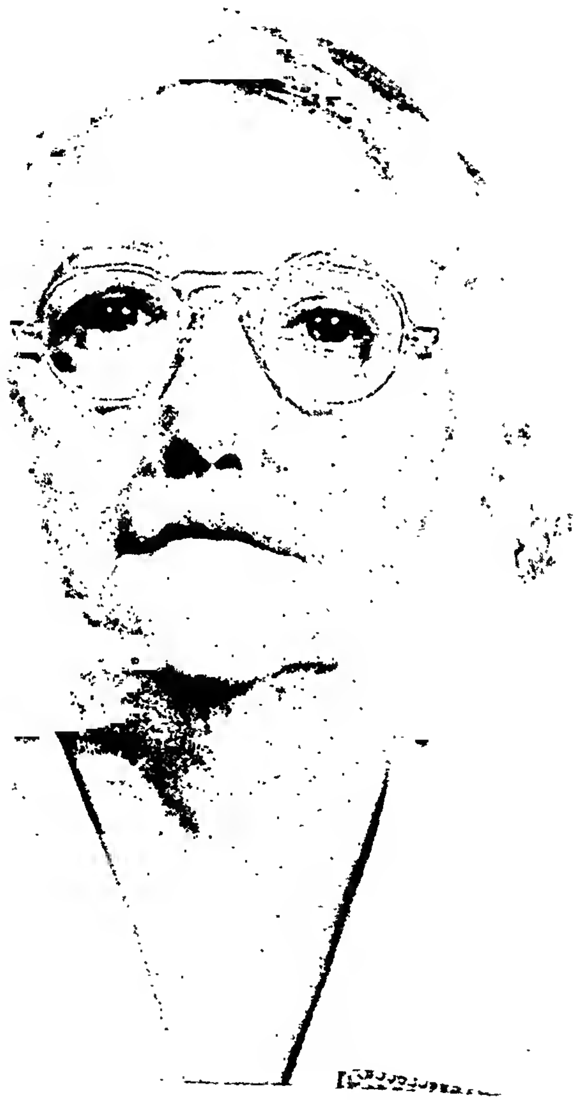


Fig. 2.—The prosthetic nose in place.

It seldom takes more than 100 c.c. of latex mixture to make the average prosthesis of an ear or nose. Therefore, the 2 to 4 per cent of zinc oxide required for this quantity can be added with an eye dropper from a stock

solution of zinc oxide ground in water to a thin paste or viscous liquid. For example, there are about 15 drops to 1 c.c.; therefore, 30 drops or 2 c.c. of this water dispersion can be added to 100 c.c. of the rubber mixture to get the proper mix for a prosthesis. This is only a rough method of figuring but it is sufficiently accurate for the purpose. If the rubber mixture contains the clay filler, the zinc oxide content can be reduced or eliminated.

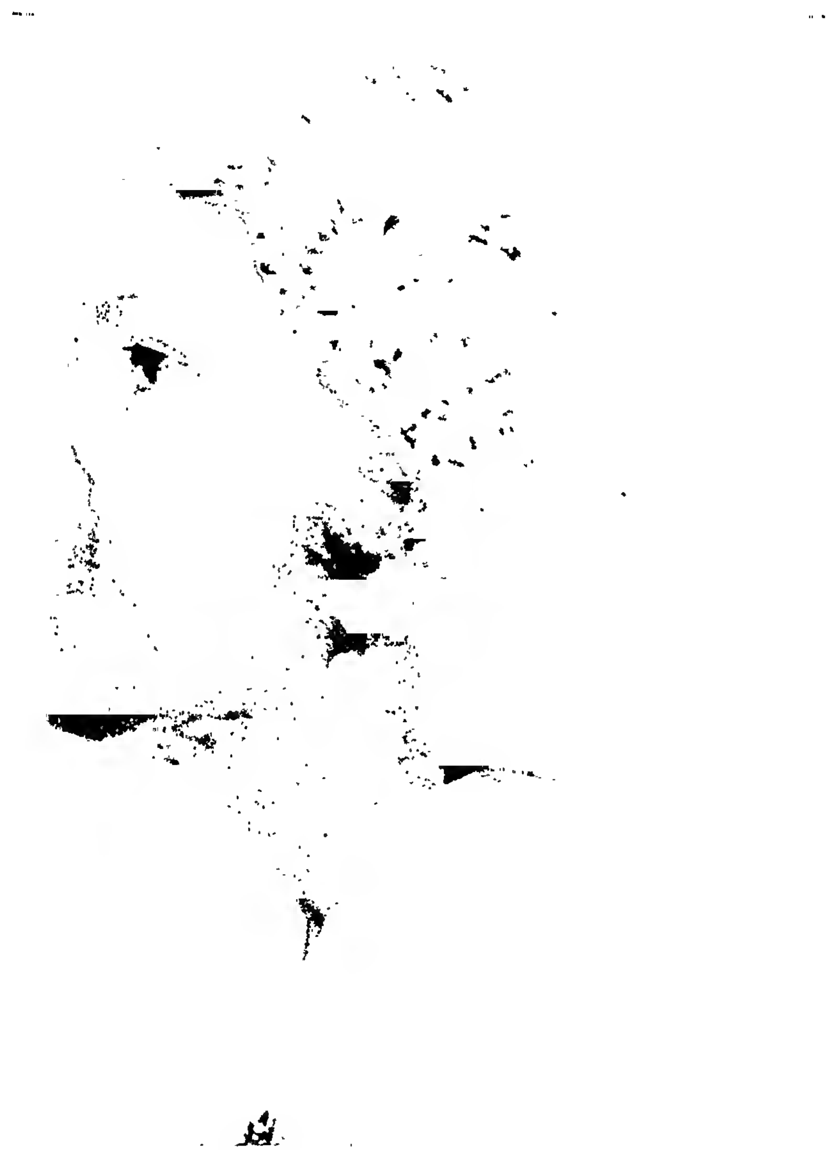


Fig. 3.—The side view of the same patient without the prosthesis.

2. *The Monochrome Base Color.*—Usually a monochrome base color is added to the latex mix to give it a general tint similar to that of the patient who is to wear the prosthetic appliance. As noted before, it is customary to have on hand various stock solutions of slightly different flesh colors because each patient's skin tones vary. This is particularly true among Negroes. As a

rule, six stock solutions suffice for Caucasians. Eight should be enough for Negroes. When a skin tone is between the color of two stock solutions, the proper color may be made by mixing the two. A dried sample of each skin tone should be attached to the neck of each bottle of stock solution, since the dried latex is different in color from the liquid latex. It is invariably darker. These samples are placed next to the skin to match the color. The prosthetist generally chooses one a shade lighter than the patient's color because other color mediums are added which eventually create the desired effect. Furthermore, all rubber prostheses tend to darken on prolonged exposure to sunlight. From time to time, over a period of months and years, it may become necessary to remake a prosthesis, and for this reason the mold of each patient should be filed away for future use.

To obtain the stock monochrome solutions previously mentioned, color in the form of pigments or dyes is added to the latex filler mixture. These pigments and dyes must be confined to certain restrictions. For example, they should not have an acid reaction to litmus paper. If they do, they will coagulate the latex into a mass or into isolated particles. They should contain no copper. If copper is present, even in small quantities, resinification of the rubber will follow. If opaque pigments are used to excess, the translucency is destroyed in the resulting prosthesis. Translucency is essential to naturalness, and for this reason transparent dyes are usually employed. Unfortunately, the average dyes available for rubber are not as permanent as pigments. Pigments are ground with water before being incorporated. Dyes are added to water until translucent solutions without any sediment are formed before incorporation. A considerably larger quantity of pigments than dyes is necessary to obtain the desired shade. When adding both pigment and dye solutions to small quantities of latex, it is advisable to use an eye dropper. The color is added a drop at a time while stirring the mixture.

In considering the brownish-red tones of Negroid skin, one may say that all three primary colors are often employed to obtain the desired effect. For this reason pigments and dyes of various shades are listed.

The following color ingredients are suitable for latex mixes:

Lithopone
Sulfide of antimony (if free from calcium sulfate)
Inactive carbon or lamp blacks, ivory black or the like
Green chromium oxide
Natural red iron oxide
Organic colors, if available in powdered or paste form

Among the water-soluble colors manufactured by E I Du Pont de Nemours & Company are the following: Pontamine fast dyes which are compatible with rubber latex and are to a great degree permanent.

Pink BL
Brown 2 RL
Blue 4 GLN
Red 8 BNL
Blue SRL
Gray BL
Orange 2 GL
Yellow NNL

These dyes are in powder form. About 20 Gm. of the powder are added to 200 c.c. of water. This mixture is added drop by drop to small quantities of rubber. The concentration of dye in water can be stronger for Negroid skin. It is difficult to give specific amounts of dye to be added to water because each person's skin varies in color, and the concentration of dye to water may vary. The prosthetist must depend on his sense of color to determine the amount of dye to be added to each latex mix or to a stock solution.

Fig. 4.—A side view of the same patient with the nose in place.

Most dye manufacturers list dyes that are suitable for incorporating with rubber. These manufacturers are listed in the Chemical Buyers' Guide Book,* which is practically indispensable to the prosthetist.

*Chemical Buyers' Guide Book, Chemical Industries, 522 Fifth Avenue, New York City.

In any event, the monochrome tint applied to the latex mix is kept somewhat lighter than is desired for the finished prosthesis, because additional oil-soluble color will be added later.

After the desired tint is chosen the mold is poured; the cast is dried in the mold and removed. The seam lines are trimmed with instruments heated over a Bunsen burner. Often a syruplike substance is thus produced. This is burnt resinified rubber and may be removed with a small piece of cloth dampened with turpentine. In fact, the prosthesis can be given a rather vigorous cleaning with the turpentine cloth. It serves to remove the tiny imperfections caused by the smallest air bubbles in the mold. The larger positive impressions of air bubbles must be cut away. The turpentine may cause the rubber cast to swell, but this will rectify as the cast dries. When one really masters the use of the tools and a knowledge of the temperature at which to use them, seam lines may be cut away with the hot tool without resinifying the rubber. Moreover, skin detail can be replaced and defects in casting removed or repaired without damage to the surface. Although depressions caused by faulty casting can be modeled out by the addition of small amounts of latex to the cast, this procedure is not always successful. It is generally better to remake the cast. The rubber cast should be washed thoroughly in soap and water after it is cleaned with turpentine.

When the prosthesis has been trimmed carefully and all defects have been corrected, it is time to use the oil-soluble dyes.

3. *Blended Shading With Oil-Soluble Dyes.*—Unvulcanized rubber is soluble in carbon disulfide with 5 per cent absolute alcohol, benzene (benzol), chloroform, benzine and oil of turpentine. Vulcanized rubber, however, only swells in these solvents, and after prolonged soaking it loses its strength to a point where it crumbles readily. Benzine being more volatile than turpentine has a quicker swelling action on the vulcanized rubber than turpentine. If oil-soluble dyes are mixed with these rubber solvents, a coloring medium is produced that will sink readily into the rubber and give permanent tints. It should be realized, however, that the dye continues to bleed into the rubber and becomes less intense. By means of this change the prosthetist is able to obtain more lifelike blended color effects on a monochrome prosthesis. In other words, skin is not a monochrome, but is made up of delicately blended areas of different shades of the same color or different colors. The same effect cannot be obtained with pigments because these are composed of microscopic particles of opaque matter which do not sink or bleed into the rubber but lie on the surface. Even if perfect blending with pigments were possible, the color would in a short time wash from the surface of the prosthesis and expose the monochrome-tinted rubber.

An oil-soluble dye applied in the same manner sinks into the rubber, and the prosthesis may be washed or even scrubbed immediately afterward. As time progresses the dye blends or bleeds further into the rubber and becomes more uniformly distributed or thoroughly blended with other shades. For this reason the prosthesis is generally colored more deeply than is desired for immediate use. The complete blending may require a few days. After several experiments, however, the worker can anticipate the effect that time will produce. It is assumed that once the solvent has evaporated from the rubber the blending will cease.

The difference between pigments and dyes can readily be detected with a microscope. For example, samples of a red pigment and a red dye are placed on a microscopic slide and studied through transmitted light. The pigments will appear as black specks and the dye will still appear red on the slide.

Fig. 5.—A side view of a patient requiring a prosthetic ear.

Oil-soluble dyes serve their most important purpose in obtaining pink-blended flushlike effects on the cheeks, lips, tips and ala of the nose, and knuckles, and blue effects of veins that lie beneath the skin.

These results are obtained by preparing a mixture of one part of turpentine and one part of benzine as a medium. Into 50 c.c. of this mixture are dissolved 10 Gm. of the oil-soluble dye. This will produce a heavily concentrated dye mixture which may be considered a stock solution. Before it is

used a drop or two of the solution is added to a few drops of the benzine-turpentine mixture. The diluted mixture is applied with a brush to tint the prosthesis at the desired places. It is advisable to try to blend at the time the color is applied, but it should be realized that the resulting blended effect takes place automatically by bleeding rather than by the artistic skill of the worker. As the colors bleed they become lighter; therefore, the tints are applied more intensely than desired in the finished prosthesis. After applying the oil-soluble dyes, the prosthesis may be left to stand or it may be washed immediately with soap and water to test the permanency of the dyes employed. If the right dyes are used and the work done properly, it will be impossible to wash off the dyes.

The following is a list of oil-soluble dyes suitable for this purpose which are manufactured by Du Pont:

Anthraquinone Blue AB Base
Anthraquinone Blue SKY Base
Anthraquinone Green G Base
Anthraquinone Iris R Base
Oil Red
Oil Black BG
Oil Brown N
Oil Yellow N
Oil Yellow
Oil Fast Yellow EG

4. *Alcohol-Soluble Dyes.*—The alcohol-soluble dyes have their place in prosthetic work, but they are not used to the same extent as those that are oil-soluble. The stock solutions of these dyes are made up in the same manner; that is, 10 Gm. of dye are added to 50 c.c. of alcohol. The intensity of the dye can be reduced as it is used by adding a few drops of dye solution to the alcohol until the desired shade is obtained. The diluted solution is applied to the prosthesis with soft brushes. Blended surface effects can be obtained. These dyes remain where they are placed and do not bleed into the rubber. In some cases it is desirable to put a thin coat of matte lacquer over the finished prosthesis, which in turn makes the alcohol-soluble dyes adhere more readily to the rubber surface. This will be discussed more fully later.

The following alcohol-soluble dyes manufactured by Du Pont are suitable for use on rubber:

Luxol Fast Red BB
Luxol Fast Red B
Luxol Fast Scarlet C

5. *Applying Hair, Artificial Eyes, and Fingernails.*—For the sake of naturalness it is sometimes desirable to imbed hair into the rubber cast. Each hair is usually inserted separately with a needle. The tip of the eye end of a small needle is filed until it is open at the end. Then the pointed end of the needle is fixed into a small piece of wood which serves as a handle. To insert the hair a pointed needle is used to pierce the rubber and the forked end of the prepared needle is used to straddle the end of the hair over the pierced rubber.

By applying pressure to the hair with the forked needle over the hair, it is forced into the previously formed hole. The strength of the rubber closing over the hair is generally sufficient to hold it in place. If the hair is placed into a hollow rubber cast, such as a masculine hand filled with a microcrystalline wax core, however, the wax will give additional adhesion in holding the hair in place.

Fig. 6.—The same patient with the ear in place.

A large area of hair as, for example, a mustache, may be applied to a male patient to hide joining lines where a prosthetic nose and lip connect to the face.

The process of setting hairs in a foundation of gauze, lace, or woven stiff silk is known as *ventilating*. The needles employed for the purpose are *ventilating needles*. These have front hooks of varying sizes, ranging from small to

large. Small ones are used to thread one or two hairs and the larger ones for more strands.

Fig. 7.—The front view of the patient without the prosthesis.

If the prosthetist does not wish to attempt this branch of the art, a professional wigmaker can supply artificial mustaches to match natural hair. Indeed, he can make them from the patient's own hair. Eyebrows may be made in the same way. Although this manner of making mustaches and eyebrows is not the most successful, it may serve the purpose. The most naturalistic eyebrows and eyelashes can be obtained from cadavers. These are cut away, tanned. and softened before use. After removal of the brow or lash the fat of the skin is dissolved out in benzine. The skin is then bleached in hydrogen peroxide or a hyperchlorate solution. It should be noted that the

peroxide will also bleach the hair and this will subsequently have to be dyed to the desired shade. The skin is then treated with tannic acid and finally softened in a weak solution of glycerin or a similar agent. Such natural eyebrows and lashes are easily obtainable by the physician and prosthetist having connection with a school of medicine.

Fig. 8.—A front view of the same patient with the ear in place.

Artificial eyelashes which may be purchased from cosmetic stores and costumers and attached to the human eyelids in order to enhance the beauty of the wearer are not suitable for prostheses because they are so long they give an unnatural appearance. If they are trimmed shorter the ends of the hairs are blunt. Natural eyelashes are tapered to pointed ends. The blunt hairs are obvious on the prosthesis.

The gauze or natural eyebrows are attached to the surface of the rubber with the same latex mix that is used to make the prosthesis. The latex mix may be applied directly to the gauze or leatherized skin with a brush, after which the mustache or eyebrow is set in place. A hypodermic syringe with a large needle may also be used to inject additional latex between the hairs on top of the gauze. By using the syringe and needle the latex can be kept from soiling the hairs.

In order to obtain the best adhesion of the latex to the set rubber, the prosthesis should first be wiped off with a cloth dipped in alcohol. This removes grease and foreign matter that may prevent the gauze or leather from sticking. It is also more natural to insert a few stray hairs into the rubber around the mustache or eyebrow.

Artificial eyes may be purchased from the larger optical and taxidermal supply stores. Those furnished by the taxidermist are globe-shaped, whereas those supplied by the optical stores are shell-shaped. The shell-shaped eyes are more expensive but better to use. The globe-shaped eyes, however, may be cut to size with a three-cornered file. In any event, the artificial eye is first matched to the real eye of the patient and fitted into the wax pattern. The wax is fitted to the patient to be sure the eye will look natural. A plaster mold is made over the eye and wax pattern. Before the rubber latex is poured into the mold the eye must be removed from the wax and replaced by itself into the mold. It may be held temporarily in its original place by a small piece of sticky wax. The mold is then closed and the latex mix poured. After the set rubber is removed, it may be necessary to trim some of the rubber where it joins the eye. This, of course, is done with hot tools. If the patient has one good eye it is logical that the glass eye incased in a rubber prosthesis will not move with the natural eye. Unfortunately, this cannot be helped but it will be less noticeable if the patient wears eyeglasses with one dark lens and one clear lens, or with two slightly tinted lenses.

If a prosthetic hand is well done it is seldom necessary to use artificial fingernails, especially with hands of men. It is advisable, however, to apply natural cellulose acetate fingernail lacquer to the rubber nails. The average woman prefers artificial nails which may be purchased from cosmeticians and five-and-ten cent stores. They are applied by simply trimming them to fit the particular rubber fingers and then sealed in place with cellulose acetate or polyvynl acetate cement. Some natural fingernail lacquers also may be used for affixing the artificial nail to the rubber fingers.

6. *Highlighting or Lacquering the Surface.*—It is not always necessary to lacquer the surface of a rubber prosthesis, but frequently this is desirable, especially to cover lips and eyelids. The lacquer on rubber often makes the rubber more receptive to cosmetics; because the rubber surface causes a "drag" on the fingers and brushes that are used in applying make-up. The wearer of a prosthesis must learn to use the lacquer as well as cosmetics. If he gets a better effect with lacquer he must clean the cosmetics and old lacquer from the prosthesis with alcohol after the prosthesis is removed. He then applies an extremely thin coat of matte lacquer before retiring for the night. The next morning the lacquer is thoroughly dry and the prosthesis is

ready for reapplying and is receptive to the make-up. Since the lacquer is alcohol-soluble, the adhesive for holding the prosthesis in place, which is also alcohol-soluble, must be applied with care to the reverse side to prevent mixing and the formation of a gum deposit on the edges. Although the lacquer is not essential and is seldom used by masculine patients, it has its place in creating special effects. It should also be realized that lacquer will eventually wash from the surface; it should not be considered as a permanent part of the prosthesis. If the prosthesis lacks translucency a matte lacquer will create an effect of translucency. A primary use of lacquer is to set or hold any surface coloring with alcohol-soluble dyes and to make the surface more receptive to the application of cosmetics, as previously mentioned. It is well known that many face creams contain an oil or grease in one form or another. These oils and greases readily attack and deteriorate rubber if used in excess. The prosthetist can use cosmetics that have no oil or small amounts of oil or grease in them, or he can make the surface of the prosthesis impervious to the action of cosmetics. *Face creams and lotions containing stearic acid may be used as a powder base, because the stearic acid does not readily attack the rubber. Therefore, vanishing creams may be used, whereas cold creams should be omitted. This subject will be discussed more fully under Cosmetics.*

If lacquer is used it should be alcohol-soluble and kept quite thin. Most lacquers can be diluted with acetone; others may be diluted with alcohol to acquire this thin consistency. This applies particularly to the polyvinyl acetate lacquers. A formula most suitable for this purpose is as follows:

	Parts by weight
Ethyl alcohol	700 Gm.
Ethylene dichloride	200 Gm.
Amyl acetate	80 Gm.
Castor oil	20 Gm.
Polyvynl acetate	100 Gm.

The liquids are mixed together and the polyvynl acetate crystals are added. The container is shaken from time to time until the polyvynl acetate is dissolved completely.

Commercial lacquers are invariably too brittle on drying for application to rubber, which undergoes considerable bending and stretching. It may also be necessary to add a small amount of castor oil to the commercial lacquer to make it more plastic on drying. If the lacquer cracks or develops other defects that make it undesirable, it may be removed from the prosthesis with a cloth dampened with alcohol. Lacquer that is not alcohol-soluble may be removed with a cloth dampened with acetone or amyl acetate.

7. *Cosmetics.*—As in the case of lacquers, cosmetics are not absolutely essential to a well-prepared and colored prosthesis. Because of the darkening effect of sunlight on rubber and the changing of skin tints as a result of the various seasons, however, cosmetics may be employed to counteract these gradual differences. Furthermore, cosmetic pastes are used to fill *join lines* to make them invisible. *Join lines* result where the prosthesis meets the natural

skin. In any event, cosmetics should be used sparingly. Any semblance to stage make-up should definitely be avoided. Opaque pastes and lotions should not be used, or the translucency of the rubber and skin will be lost and produce an unnatural appearance. The following general remarks on the application of prostheses and make-up to the face should be studied carefully. It should be remembered that there are a variety of facial prostheses, such as eye sockets, noses, ears, and depressions or scar casts.

1. The face is washed with a mild soap. If the patient has a tendency toward oily skin, an astringent should be used after washing. Practically any astringent is suitable for this purpose. A satisfactory one may be prepared as follows:

Alcohol	1,000 c.c.
Menthol	4 Gm.
Glycerin	50 c.c.
Water	1,300 c.c.
Perfume	q.s.
Color (vegetable dye)	q.s.

The menthol is dissolved in the alcohol and the other ingredients are added in the order mentioned.

2. The adhesive is placed on the side of the prosthesis that comes in contact with the skin surfaces. It should not extend to the front or back beyond the area that is made to fit the face. I have found the following formula suitable for this purpose:

Gum mastic or colophony	175 Gm.
Ether	200 c.c.
Alcohol	200 c.c.
Castor oil	10 c.c.

The gum mastic is placed in a bottle; the castor oil is mixed with the alcohol and then added to the gum mastic. Finally, the ether is poured in and the bottle is shaken well. In a day or so the gum mastic will be dissolved completely. Impurities in the mastic, such as dirt or pieces of bark, settle to the bottom. The clear liquid is poured off and put into smaller bottles to be supplied to the patient. The gum mastic in this formula is the adhesive agent. The ether is for quick drying and the alcohol is for slower drying. Some patients prefer to use ether as the solvent for gum mastic, because they can put the prosthesis in place the moment the adhesive is applied. Others prefer to use alcohol only, since it gives them a minute or so to wait before putting the prosthesis in place. The castor oil is used to prevent the mastic from crystalizing on drying. Colophony (rosin) can be substituted for the gum mastic. I know of two cases that developed a rash from the use of gum mastic. In such a case another gum should be substituted.

3. After the prosthesis is in place a pastelike putty should be applied over the join line where the prosthesis comes in contact with the human skin. This is particularly necessary for noses and eye sockets, but is not essential in prosthetic hands. The putty is used sparingly on small artificial portions of

the ear to cover the join line where the lobe or top curvature meets the natural ear. If an entire ear is made the join line can be planned at the least conspicuous place next to the head. A join line in the front of the ear should be made invisible.

A theatrical nose putty can be purchased from drug stores. This material is difficult to use, however. A far more satisfactory product can be prepared as follows:

Be Square special wax, 160 to 165 m.p., amber (Bereco Oil Co., Tulsa, Okla.)	20 Gm.
Mineral oil	5 c.c.
Rosin (water white)	20 Gm.
Talc	20 Gm.
Red color (oil-soluble)	q.s.
Perfume (oil or alcohol-soluble)	q.s.

The mineral oil and wax are melted together; the rosin is then added and the mixture stirred over a low fire until completely melted. It is removed from the fire and the talc added. The mixture is stirred until cold. If too sticky more talc is added while cold, and the mixture is kneaded like dough until it will not stick to the fingers. A drop of diluted oil-soluble red color and a drop of perfume are added and the kneading is continued until the correct color is obtained. The wax gives plasticity to the composition, and the rosin causes adhesiveness. The mineral oil is used to overcome the dryness produced by the talc and to improve the pliability. The talc counteracts the extreme stickiness of the mixture and reduces the excess translucency. This putty should be of a consistency that bends readily without cracking as the face assumes different expressions. For winter use, a few additional drops of mineral oil may be added. The color should be of a shade and quantity to suit the individual. This is of no great consequence, however, since a stock supply can be made up without the color. The color can be added to a small amount of the stock supply while kneading the putty in the fingers. From time to time the putty is held next to the patient's skin for matching. When the correct tint is reached the putty is given to the patient for use.

This mixture is employed to cover join lines in the following manner. The fingers are dipped in water to keep the putty from sticking to them. A small piece is rolled between the fingertips until it is cylindrical and about the size of a toothpick. This is laid over the join line and pressed into place with a wet finger. It is then smoothed out until the prosthesis blends with the face and the join line disappears.

4. After the join line is covered a skin lotion or vanishing cream may be used as a powder base to cover both the prosthesis and the face. This should not be of an oily nature and should be applied sparingly. A small amount is dappled on the forehead, cheeks, and chin, then blended with the fingertips into the skin and prosthesis until the lotion disappears.

The following formulas devised by the Atlas Powder Company are most satisfactory for use with prostheses for powder bases, vanishing cream, skin lotion, and similar preparations:

	Ingredients	Formulas 1	2	3	4
A.	1. Arlex	3.0	8.0	5.0	2.5
	2. Potassium carbonate	0.7		0.7	
	3. Potassium hydroxide		1.0		
	4. Triethanolamine				1.7
	5. Water	76.4	64.1	45.1	70.9
	6. Preservative	q.s.	q.s.	q.s.	q.s.
	7. Stearic acid	15.0	20.0	10.0	3.5
	8 Lanolin	0.5	1.5	0.5	2.0
	9. Mineral oil	2.0	2.0	35.3	15.0
B.	10. Mannitan Monooleate (Atlas G-954)	2.0	1.0	1.0	2.0
	11. Mannitan Monostearate (Atlas G-908)			2.0	2.0
	12. Spermaceti		2.0		
C.	13. Perfume	0.4	0.4	0.4	0.4

METHOD OF MANUFACTURE

Warm A and B separately to 75 to 80° C.
Add B and A slowly and with thorough agitation.
Add C at 50° C.
Note: Stir No. 1 and No. 2 intermittently, and when body has increased, turn over by hand. No. 1 cream may be poured directly into jars at 58° C.
No. 4 cream must be passed through colloid mill or homogenizer while still fluid.
No. 3 cream should be stirred until cold, set aside over night, and remixed the following morning.

No. 1, Vanishing Cream
No. 2, Hand Cream
No. 3, All Purpose Cream
No. 4, Liquid Cream

MODIFICATIONS

For modification of the above formulas the following will serve as guides:

Foundation Cream.—Formula No. 2. Add 2 per cent titanium dioxide.

Powder Cream.—Formula No. 2. Add 5 per cent titanium dioxide and color lakes.

Emollient Skin Cream.—Formula No. 3. Increase lanolin to 5 per cent or as required.

Liquid Cleanser.—Formula No. 4.

Hand Lotion.—Formula No. 4. Reduce mineral oil to not over 5 per cent, increasing water by like percentage.

Liquid Powder Base.—Formula No. 4. Reduce mineral oil to 5 per cent as above.

5. After the powder base is applied and dried, moist rouge or powder cake rouge may be blended into both the skin and prosthesis at the proper places. The moist or cream rouge serves best for this purpose because the dry cake rouge does not spread evenly over rough or hairy skin and enlarged pores Some rouges have a tendency to permanently dye the rubber. If the rouge

used cannot be removed from the prosthesis with alcohol it should not be considered for prosthetic work. Rouge also can be used to good advantage on the ala of a prosthetic nose or the top curvature and lobe of a prosthetic ear if the prosthesis lacks color at these points or if fugitive colors were used in making the prosthesis. The rouge should be applied sparingly and blended with the fingertips. In making up a prosthesis the tendency should always be toward naturalness without in any way leaning toward the theatrical. The main object is to prevent a "made-up" appearance and to avoid attracting undue attention to the face.

6. Any face powder of suitable shade and texture can be used over a proper powder base. It should be dusted on lightly and blended over the whole with a soft powder puff. It, too, should be applied sparingly to prevent an opaque cast to both natural and artificial skin. Powder applied to the skin has a tendency to kill the natural sheen and high lights. Often it is desirable to restore the natural qualities of the skin. A cold moist towel gently pressed against the face and prosthesis will set the powder and at the same time restore natural skin characteristics. This is especially desirable for a male patient. The powder aids greatly in hiding properly filled join lines.

7. Lipsticks or paste can be used in the usual manner over both rubber and natural lips, but should not be used in excess, since the grease content may cause the rubber to deteriorate unless it is lacquered. Indelible lipsticks should be avoided because they stain the rubber to such an extent that the color cannot be removed. It is good practice to test a lipstick on the back of a prosthesis to see if it can be removed properly before using it on the lips.

8. Eyebrow pencils can be used if desired on rubber prosthesis with hair. Such prostheses should be made to withstand washing with mild soap and water and an occasional cleaning and disinfecting with alcohol.

9. Brilliantine and similar hair preparations can be applied with a tooth or mascara brush on the hair of a prosthesis. Such mixtures should be used with care and very sparingly, however. An excess of oil in the artificial hair collects dust and foreign particles. This necessitates frequent cleaning, resulting in premature deterioration of the artificial hair, and replacement becomes necessary.

Editorial Announcement

Previously it has been customary to end the volumes of THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE with the September issue. A change has been made, however, by which the future volumes will conform to the calendar year, beginning with the January issue and ending with that of December. The present volume 28 will, therefore, continue through the December number, and the index which has been omitted from this number will appear instead in that issue.

Editor.

The Journal of Laboratory and Clinical Medicine

Vol. 28 October, 1943 No. 13

CLINICAL AND EXPERIMENTAL

THE PHYSICS OF SOUND WITH PARTICULAR RELATION TO EXAMINATION OF THE PATIENT

Earle M. Chapman, M.D.,* and Avram Goldstein,† Boston, Mass.

INTRODUCTION

THE application of the principles of physics to the examination of the patient was introduced by Skoda and Hoppe[1] 100 years ago. Since that time their ideas have been amplified and experimentally verified.[2] We believe, however, this knowledge has been neglected in current teaching and for this reason we are presenting these ideas, not original, which are necessary for the student's appreciation of the fundamentals of physical diagnosis. Montgomery and Eckhardt's[3] admirable work clarified the physics of breath sounds, and herein we hope to accomplish the same for the phenomena of percussion and tactile fremitus. For clarity we shall present this material in three steps:

1. Sound waves—four qualities: intensity, pitch, duration, and quality (timbre).
2. Sound waves—four fates: diffusion, absorption, reflection, and resonance.
3. Application of the physical principles to tactile phenomena and percussion.

I

Sound is the result of rhythmic vibrations of the air striking upon the ear. The range of audible sound is from 16 to 36,000 vibrations per second. The range of musical tones is from 40 to 4,000. Sound waves can be represented diagrammatically to show the *four qualities* of sound: intensity, pitch, duration, and quality, as follows:

*Instructor in Medicine, Harvard University.
†Third Year Student, Harvard Medical School.
Received for publication, February 1, 1943.

1. *Intensity.*—In this we are concerned with the *amplitude* of the vibrations or the total excursion of the mass of air moved. In Fig. 1 wave length is the same in 1A and 1B, but amplitude differs. The tones shown are identical, but since 1A is greater in intensity than 1B it is a louder note.

2. *Pitch.*—In this we are concerned with *frequency* (vibrations per second) that differentiates one pitch from another. A deep note is one of low frequency, Fig. 1, 2A; a higher note is one of many vibrations per second, 2B.

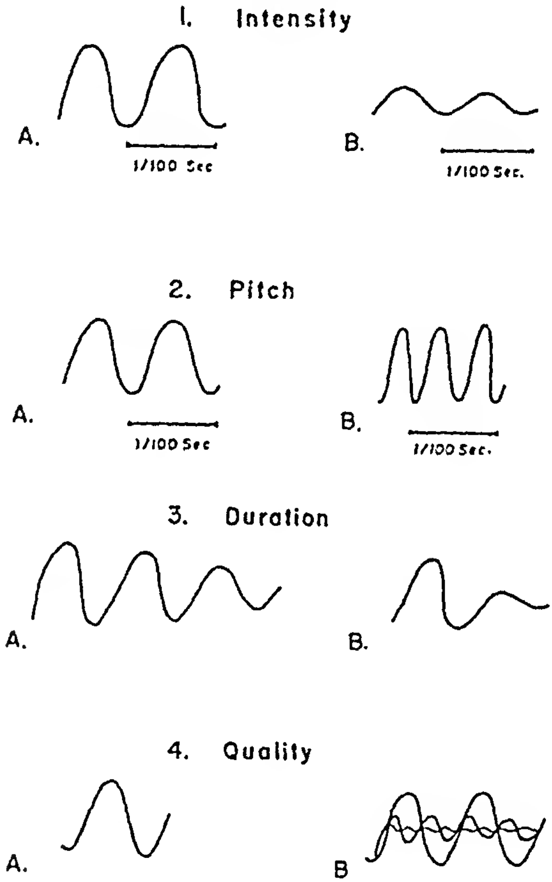


Fig. 1.

3. *Duration.*—All sounds diminish in intensity with time; they "die out." In Figs. 1 and 3, the tones are identical except that the duration of A is greater than that of B. Once the initial disturbance creating the sound no longer exists, the sound will persist for a shorter or longer time depending on a number of factors. The chief factor determining duration is the "sympathetic vibration" of a large mass of elastic material, usually air, which reinforces the original sound. For example, the plucking of a violin string alone produces a short sound, but with this string stretched over the sounding box, a large mass of air is set into vibration and a sustained tone results.

4. *Quality (timbre).*—The concept of quality is exemplified by the dissimilar tones of a violin, a voice, and a flute, all sounding the same note at the same intensity for the same length of time. Quality depends upon the presence or absence of overtones. When a violin string or a column of air vibrates at a certain frequency, the whole string or air column is in vibration as a unit.

The string or air column may vibrate in halves, giving a note of twice the frequency; or in thirds, fourths, and so on, and these various notes are heard superimposed upon the fundamental tone. What we usually call "richness" of tone is the presence of many overtones, all bearing a direct harmonic relationship to the fundamental, that is, all simple multiples of the fundamental. In Figs. 1 and 4, A is a pure tone, while B is rich in overtones.

II

Of what importance are these concepts when one is studying sound traveling through the body tissues? In tactile fremitus the vibrations originate within the body and travel outward to the palpating hands of the examiner; in percussion some of the sound waves travel from the percussing finger toward the interior of the body, where they are amplified or not, depending upon the properties of the substances underlying their point of entry; in auscultation the sounds arise within body tissues and travel toward the surface and the observer's ear.

Four fates lie ahead of such sound waves traveling through body tissues.

1. *Diffusion.*—The vibrations may set into motion tissues or air lateral to their direction of motion. Such lateral vibrations never reach the hand or ear of the examiner, since they gradually subside through diffusion in several directions.

2. *Absorption.*—This is the complete disappearance of sound vibrations, the energy of which is transformed into heat in the substance in which the sounds are damped out. Absorption must really be considered together with its opposite, transmission, since the properties of the medium determine whether sound will be transmitted or absorbed. A very elastic substance like air enclosed in a space with elastic walls (lung) transmits most of the sound waves passing through it. On the other hand very inelastic material (soft tissue, muscle) absorbes most of the vibrations.

3. *Reflections.*—The use of this term in connection with sound waves is similar to its use in optics. For reflection of sound waves to occur there must be two media meeting at an interface; reflection is favored by a large difference in density between the media. The best example of this is pleural fluid, which acts as a reflecting layer so that the vibrations of fremitus cannot reach the palpating hand nor can those of the percussion blow reach the underlying lung.

4. *Resonance.*—This term is used here to describe a physical principle, and not in its more specific meaning as one of the percussion sounds. The principle of resonance is that of tone reinforcement (sympathetic vibration) mentioned previously. If the physical properties of a large mass of tissue or enclosed air are such that it can be set easily into vibrations at a particular rate, then a sound of that frequency in the vicinity will cause the entire mass to vibrate. In this way the feeble sound of finger striking finger is amplified, as illustrated by resonance heard on percussion of the normal thorax or the tympanitic note heard over air-filled viscera.

III

We wish to stress the practical application of these fundamental concepts to the actual procedures by which we elicit and interpret the percussion note and tactile phenomena.

PERCUSSION

The purpose of percussion is to determine the physical properties of tissues by directing sound waves into them and observing the note which results. Since the object is to transmit the percussion blow to the underlying structures, *the pleximeter finger should be pressed very firmly* against the body wall. The plexor finger should strike a *sharp, staccato blow* and be immediately withdrawn to allow the fullest opportunity for vibrations to occur; a laggard blow may damp the vibrations. To offset diffusion (lateral radiation), however, *the percussion blow should be struck perpendicularly* to the surface, so that the resulting note will be affected only by the structures directly beneath. Skoda emphasized that percussion is chiefly *comparative*, and for comparing different areas the *intensity of the blow must be consistent.*

The question of the strength of the blow is somewhat dependent on the individual's reaction. For example, if percussion is performed too forcibly, organs cannot be well outlined because radiating sound waves impinge upon nearby organs or air spaces and confuse the resulting audible tone. This spreading occurs even under the best of conditions, as shown by the difficulty in outlining the right lower border of the heart. On the other hand, to outline masses lying at some depth, a rather heavy blow is required; for instance, the dulness over the left lower lung secondary to cardiac enlargement[4] is best appreciated with a forceful percussion.

It is evident that the percussion note will lie between two broad extremes: a large mass of air may be set into sympathetic vibration and so reinforce the percussion blow, or the sound may be reflected back at once by rigid, nonelastic structures, and only the dull thud of the percussion stroke will be heard. For convenience the sounds elicited are classified into four main gradations between these extremes. These will now be described in terms of their acoustic properties.

1. *Tympany* (Fig. 2, 1.).—Tympany is a definitely musical note, of pure pitch and with few overtones, produced when a mass of air vibrates in sympathy with the percussion note. It is best obtained normally over the region of the cecum or stomach. Whenever heard, it is an indication of the presence of air. Hence in pneumothorax, extreme emphysema, and over large cavities, it is a pathologic sign. Tympany can be approached experimentally by percussion over the normal thorax in full inspiration; such hyperresonance may also be simulated by percussion of a loaf of fresh bread or a feather pillow.

2. *Resonance* (Fig. 2, 2).—Resonance is a note of deeper pitch, richer in overtones and somewhat shorter in duration than tympany. It is the note heard over the normal lung. The elastic tissue of the alveolar walls tends to localize the vibrations and favors the setting up of these overtones by the separate vibration of many different air masses (alveoli).

3. *Dulness* (Figs. 2, 3).—Dulness is higher pitched and of shorter duration than resonance. A dense tissue underlies the pleximeter so that a comparatively small amount of air is set into vibration and the percussion note is only slightly reinforced. Dulness is the normal note over the heart, while over the lung it indicates consolidation, collapse, fluid, fibrosis, congestion, or a thickened pleura or chest wall. *Dulness in place of resonance is always a sign of disease.* "Ubi sonus est altior, ibi est morbus," as Auenbrugger said in 1755.[5]

4. *Flatness.*—Flatness is the epitome of dulness. The pitch is very high; the note is entirely nonmusical, rather a noise than a tone. The duration is minimal; the intensity is minimal; and the pleximeter finger even feels the sense of resistance. There is no reinforcement or sympathetic vibration whatever, and the note heard is simply that of the percussion blow itself. Flatness is the note heard normally over the thigh and pathologically over fluid and very thick tissue.

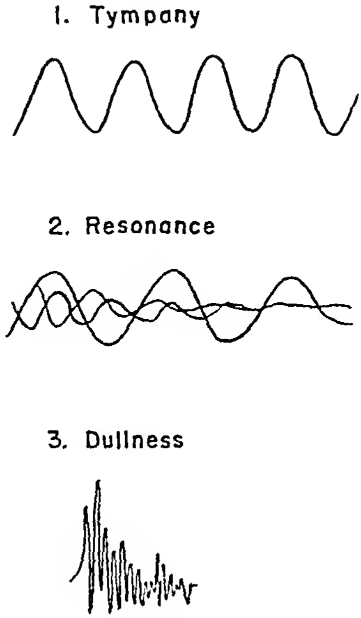


Fig. 2.

TACTILE PHENOMENA

To understand the principles underlying tactile fremitus, we drew the analogy of the human vocal cords, trachea, lungs, and thorax to a wind organ. The vocal cords correspond to the stops; the trachea to the pipe; the lungs to the bellows; the thorax to the resonator. The patient phonates in a full, deep voice, while the examiner's hands are placed on the thorax. Vibrations produced in the larynx pass down the air passages, are reinforced by sympathetically vibrating lungs, and are then transmitted through the pleura and chest wall to the examiner's hands. Fremitus therefore depends upon all the anatomic structures named, their condition, and particularly their physical properties with respect to the passage of sound waves (Fig. 3). Tactile phenomena arising from the movement of the heart, blood, pleural surface, or other viscera will follow the same principles in physics.

1. *Larynx.*—The vibrations of a bass voice correspond closely to the fundamental tone of the normal lung which they set into sympathetic vibration. The higher the pitch of the voice, the weaker are the vibrations one feels. This simple matter of voice quality must always be considered in evaluating fremitus. In weak, high-pitched voices one may feel little or no fremitus. In children, however, the higher-pitched voice corresponds to the fundamental tone of their lung and therefore fremitus is easily felt.

2. *Air Passages.*—The trachea and bronchi must be patent, or the vibrations will not pass freely and fremitus will be diminished or absent as in bronchial obstruction or in collapse of the lung with a closed bronchus. In atelectasis of the lung with an open bronchus the collapsed tissue may rest against the chest wall and so fremitus may be increased.

3. *Lungs.*—In consolidation fremitus is increased, because the solidified lung presents fewer air-tissue interfaces (alveolar septa) at which reflection can occur; and because solidified tissue vibrates more rapidly than does the normal elastic lung, thereby reinforcing the comparatively rapid vibrations of the spoken voice. In emphysema tactile fremitus is decreased because of larger air spaces and inelastic septa, which allow more absorption and diffusion of vibrations before they reach the palpating hand.

4 and 5. *Pleura and Pleural Space.*—These merely transmit the vibrations from lungs to chest wall. Any absorbing or reflecting layer will prevent such transmission; thus in thickened pleura, pleural effusion, and pneumothorax, fremitus is diminished or absent.

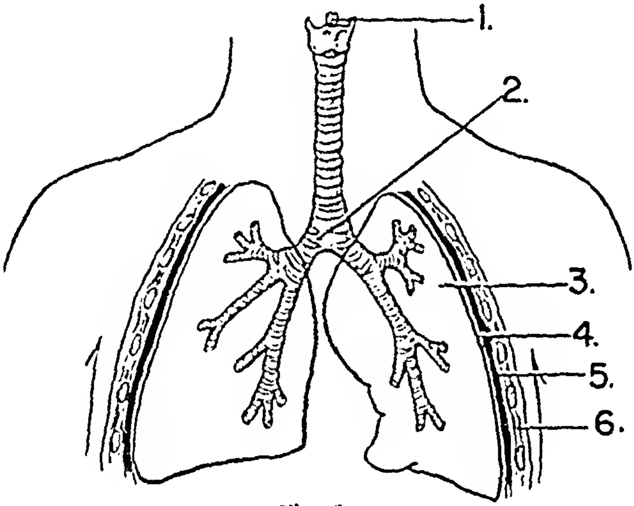


Fig. 3.

6. *Chest Wall.*—Fremitus is decreased over a fat or muscular chest wall because of absorption and diffusion; it is increased over a thin and bony one. One should also remember that for anatomical reasons fremitus is normally greater in certain locations than others. These locations are: (a) Posteriorly, over the right upper lobe, because the trachea lies in direct contact here with the lung apex, while on the left it is separated by the aorta, esophagus, and spongy connective tissue; (b) posteriorly low in the right interscapular area, because the right bronchus which is the direct continuation of the trachea points at this region; (c) in the interspaces rather than over the ribs. It is worth noting that the trachea does not run up and down, but is very oblique, running from the larynx posteriorly to the bifurcation and thence to each lung hilus, very near the posterior boundary of the mediastinum.

CONCLUSION

We offer this review as a guide to the student seeking to acquire skill in the physical examination of the patient. Such skill is not acquired by memo-

rizing lists of diseases and their physical signs, but rather by understanding the physics of sound in relation to normal and diseased tissues.

REFERENCES

1. (a) Skoda, Joseph: A Treatise on Auscultation and Percussion, translated by W. D. Markham, London, 1853, Highley & Son.
   (b) Hoppe, F.: Zur Theorie der Percussion, R. Virchow Archiv. 6: 145, 1854.
2. Martini, Paul: Studien uber Perkussion und Auskultation, Deutsches Arch. f. klin. Med. 139: 65, 257, 1922.
3. Montgomery, C. M., and Eckhardt, E. A.: Pulmonary Acoustic Phenomena, Tenth Annual Report of the Henry Phipps Institute, 1915.
4. Chapman, E. M., and Anderson, R. G.: Aids in Physical Diagnosis: Hitherto Undescribed Signs of Compression of the Left Lower Lobe Due Chiefly to Cardiac Enlargement. Unpublished data.
5. Auenbrugger, Leopold: Inventum Novum ex Percussione Thoracic Humanis, Vienna, 1761. Translated by John Forbes, M.D., London, 1824, Johns Hopkins Press, 1936.

EFFECT OF TUNGSTEN METAL DIETS IN THE RAT*

F. W. KINARD, PH.D., AND JOHN VAN DE ERVE, M.D., CHARLESTON, S. C.

FEW reports on the toxicity of tungsten metal are recorded in the literature, although the substance has been used as a substitute for bismuth salts in roentgenologic examination of the human gastrointestinal tract.

Bernstein-Kohan[1] killed and examined a rooster three days after he had fed the fowl 438.2 mg. tungsten daily for eight days. No trace of tungsten was found in the kidneys or the liver, but extravasations of blood were found in the mucous membrane of the duodenum, jejunum, and cecum.

Kruger[2] recommended powdered tungsten metal as a substitute for bismuth salts in roentgenologic examination of the gastrointestinal tract. He observed no ill effects in patients given orally 25 to 80 Gm. This followed preliminary work in which 3 rabbits were fed 3 to 5 Gm. of the powdered tungsten metal and 2 dogs were fed 10 to 20 Gm. In no case was there evidence of toxic action.

Waltner[3] incorporated 2 per cent tungsten metal in the McCollum stock diet and fed this to 1- to 2-month-old rats. He stated that growth was somewhat poor, but made no mention of control animals or of the duration of the experiment.

The present work was carried out in order to determine the effects upon the growth of the rat of extended feeding of various levels of powdered tungsten metal. These experiments should give evidence of any marked toxic action of the tungsten.

EXPERIMENTAL

Thirty-eight-day-old animals were chosen from the College colony of mixed Wistar albino and Minnesota piebald rats. Each group was made up of at least 5 females and at least 5 males with the sexes caged separately.

For the experimental diet, powdered tungsten metal† was mixed into the control diet of ground Purina dog chow. Containers of diet remained in the

*From the Department of Physiology, Medical College of the State of South Carolina.
Received for publication, February 3, 1943.
†General Electric Company, Cleveland Wire Works, Euclid, Ohio.

cages at all times, and a record was kept of the consumption. The rats were weighed at intervals of ten days for a period of seventy days.

At the end of seventy days, the animals were sacrificed and the gastrointestinal tracts were examined grossly.

RESULTS

In Table I, averages for 5 males and for 5 females are listed separately for the control and the three experimental diets. The gain in weight, the diet consumption, and the tungsten (W) consumption, each expressed in grams, are listed for each successive 10-day period. The values for the entire 70-day experimental period are then summed.

TABLE I

DIET CONSUMPTION AND WEIGHT GAINED PER RAT (AVERAGED FOR SUCCESSIVE TEN-DAY PERIODS*)

		CONTROLS		2% W DIET		5% W DIET		10% W DIET	
		MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
10 days	Gain	38	26	45	41	46	35	47	29
	Diet	120	110	130	110	190	130	140	120
	Tungsten	0.0	0.0	2.6	2.2	9.5	6.5	14.0	12.0
20 days	Gain	36	27	28	22	45	20	37	20
	Diet	110	120	130	100	160	130	150	110
	Tungsten	0.0	0.0	2.6	2.0	8.0	6.5	15.0	11.0
30 days	Gain	40	21	37	15	39	22	41	17
	Diet	140	110	130	100	150	120	150	110
	Tungsten	0.0	0.0	2.6	2.0	7.5	6.0	15.0	11.0
40 days	Gain	24	13	20	12	23	10	27	9
	Diet	150	120	140	100	160	110	160	110
	Tungsten	0.0	0.0	2.8	2.0	8.0	5.5	16.0	11.0
50 days	Gain	22	14	21	8	27	10	24	11
	Diet	130	110	140	110	150	110	150	100
	Tungsten	0.0	0.0	2.8	2.2	7.5	5.5	15.0	10.0
60 days	Gain	14	3	9	11	17	7	11	4
	Diet	140	100	140	110	140	110	150	100
	Tungsten	0.0	0.0	2.8	2.2	7.0	5.5	15.0	10.0
70 days	Gain	9	6	12	5	10	3	11	3
	Diet	130	100	140	110	140	110	140	100
	Tungsten	0.0	0.0	2.8	2.2	7.0	5.5	14.0	10.0
Total	Gain	183	110	172	114	207	107	198	93
Total	Diet	920	770	1050	740	1090	820	1040	750
Total	Tungsten	0.0	0.0	21.0	14.8	54.5	41.0	104.0	75.0

*All values expressed in grams.

No extravasations of blood were evident in the mucous membrane of either the small or large intestine.

DISCUSSION

Over the seventy-day period, the three tungsten diets were without appreciable toxic effect on the males, if growth is used as the criterion. The males upon the 5 per cent and 10 per cent W diets actually showed greater total gains than did the control animals, while the males on the 2 per cent diet gained 6 per cent less than the controls. During the experimental period, each male on the 2 per cent diet consumed an average of 21.0 Gm. of tungsten; on the 5 per cent diet, 54.5 Gm. of W; and on the 10 per cent diet, 104.0 Gm. of W.

The female rats on the 2 per cent and 5 per cent diets showed normal gains despite consumption of 14.8 Gm. and 41.0 Gm. of W, respectively, over the

70-day feeding period. In the females on the 10 per cent diet each consumed a total of 75.0 Gm. of W and gained 15.4 per cent less than the controls. The 75.0 Gm. of tungsten could not be utilized for nutritional purposes, and since these rats consumed even less diet than did the controls, the reduced intake of nutritient material partially, at least, explains the smaller gain. It nevertheless is clear that the 2 per cent, 5 per cent, and 10 per cent W diets did not exhibit marked toxic action.

No extravasation of blood were evident in the mucous membrane of the small or large intestine. This differs from the findings of Bernstein-Kohan, but it is probable that the metal used in the present study was more finely and more uniformly powdered than that used by Bernstein-Kohan and thus mechanical injury was avoided.

It should be pointed out that in this study the growth of the rat is used as the main criterion of toxic action. It is not maintained that the growth study alone will reveal all possible toxic action.

SUMMARY

The feeding of 2 per cent, 5 per cent, and 10 per cent tungsten metal over a period of 70 days is without marked effect upon the growth of the rat, as measured in terms of gain in weight.

Each male of the 10 per cent diet consumed an average of 104.0 Gm. of W during 70 days and maintained normal growth. The females on this diet gained 15.4 per cent less than the controls and consumed 75.0 Gm. of W. This was the greatest variation from normal that was observed.

No extravasations of blood were observed in the gastrointestinal tract.

REFERENCES

1. Bernstein-Kohan, J.: Ueber die Wirkungen des Wolframs, Arb. D. Pharmk. Inst. zu Dorpat. 5: 42, 1890.
2. Kruger, R.: Kolloidales Wolfram als Ersatz fur Wismut bei Rontgenaufnahmen des Magen- und Darmkanals, Munchen. med. Wchnschr. 59: 1910, 1912.
3. Waltner, K.: Über die Wirkung einiger Metalle, Arch. f. exper. Path. u. Pharmakol. 141: 123, 1929.

SULFATHIAZOLE AND STAPHYLOCOCCUS ANTITOXIN IN EXPERIMENTAL STAPHYLOCOCCIC INFECTION*

Edward Steinfield, M.D., Michael Brylawski, M.D.,† and Carrie B. Nash, M.T., Philadelphia, Pa.

BEFORE the advent of chemotherapy with sulfonamide derivatives such as sulfathiazole and sulfadiazine, the literature on staphylococcic bacteremia had concerned itself mainly with forms of serotherapy and the use of bacteriophage. The recognition of exotoxins which were capable of producing many of the toxemic effects of staphylococcic suppuration prompted the use of sera containing adequate amounts of neutralizing substances against these toxins. These antitoxins have been found particularly useful when toxemia rather than bacteremia has been the important source of morbidity. As Spink, Hansen, and Paine[1] have pointed out, there are certain factors which vitiate the efficacy of such sera. These are in brief: (1) The organism may have invasive properties without much exotoxin production; (2) the exotoxin if produced may be neutralized by the serum without checking the inroads of the bacteria; (3) the usual serum though containing antihemolytic factors does not contain antileucocidin substances. We agree in the main with these cogent objections with the reservation that these methods served their purpose in the past. This was particularly so in suppurative processes with toxemia and only intermittent bacteremia. The effectiveness of serum was probably enhanced when other adjuncts were employed, such as bacteriophage, blood transfusion, surgical drainage, and general supportive measures. We have had no experience with Julianelle's rabbit serum, which contains specific carbohydrate precipitins.

The development of specific chemotherapy has changed the whole aspect of this problem, since it has provided the research worker and clinician with radicals capable of bacteriostatic effect even with the disadvantages inherent in all these compounds of lessened penetration into purulent foci. Spink et al.[1] report on the results of chemotherapy with sulfapyridine and sulfathiazole in staphylococcic bacteremia. Six of ten patients treated with sulfapyridine recovered; of nineteen patients treated with sulfathiazole, one died; one other patient died subsequently of myelogenous leucemia. They consider sulfathiazole superior to either sulfanilamide or sulfapyridine and emphasize the necessity of appropriate surgical drainage of abscesses. The use of combined methods of treatment is advocated by Skinner and Keefer,[2] based on a study of 122 cases of staphylococcic bacteremia. They recommend antitoxin, blood transfusion, surgical drainage of foci, and chemotherapy (sulfapyridine or sulfathiazole). They believe that recovery of the host depends on the presence of three conditions: (a) active and viable leucocytes and phagocytes; (b) antibacterial antibodies; and (c) antibodies to the toxins and leucocidin. Recently Kolmer and

*From the Clinical Laboratories of the Jewish Hospital, aided by a grant from the Sidney A. Aloe Research Fund.

†Captain, U. S. Army Medical Corps.

Received for publication, February 10, 1943.

Brown[4] have reported on experimental chemotherapy and chemoserotherapy in staphylococcic infection. Mice were infected by intravenous injection of broth cultures; in addition, they also inoculated rabbits with broth cultures intravenously once a day for eight days in order to create a septicemic state analogous to clinical infection. Intradermal infection of rabbits was also produced to create spreading lesions due to toxin formation. They concluded that there was a slight but definite increment of therapeutic result with sulfathiazole and serum contrasted with sulfathiazole alone.

We have investigated the use of combined drug and serum therapy of experimental staphylococcic infection in a small but statistically significant group of mice. Sulfadiazine had not yet been described when the experiments were started, and we therefore confined our efforts to sulfathiazole alone and sulfathiazole combined with staphylococcus antitoxin.

TABLE I

CHEMOTHERAPY AND CHEMOSEROTHERAPY OF EXPERIMENTAL STAPHYLOCOCCUS INFECTION

TREATMENT	SINGLE DOSE (DRUG) GM./KG.	DAY OF DEATH
None (control)	—	2 5 2 7 1 8 4 8 1 12 5 12
Sulfathiazole	1.0	5 6 7 18 9 S 12 S 14 S 15 S
Sulfathiazole and serum	1.0 0.5 c.c. of 1:2	4 17 4 18 6 S 10 S 11 S 13 S
Serum	0.5 c.c. of 1:2	3 5 5 6 7 7 17

S = survived.
Ringed numbers are animals showing renal abscesses.

METHODS

Male albino mice were injected intravenously by the dorsal vein of the tail with broth suspensions of a virulent strain of *Staphylococcus aureus*. Approximately 800 million organisms were given in volumes varying from 0.2 to 0.3 c.c. The strain was supplied to us through the kindness of Dr. G. W. Barlow of the Winthrop Chemical Co. and was of proved virulence to mice. We verified its pigment, hemolysin, and coagulase-producing properties. Sulfathiazole in a suspension of acacia was deposited into the stomach transorally with a blunt needle and syringe within 1½, 7, and 24 hours, then once daily thereafter. The dosage of the drug is given in Table I, and the usual bulk of material was 0.4 c.c. The absorption of the drug was proved by levels obtained on sacrificing a number of control mice and pooling the heart's blood. We were able to demonstrate a level of 15 mg. per 100 c.c. in 1½ hours, which, though not so high as figures obtained by van Dyke et al.,[5] were considered fairly adequate. Staphylococcus antitoxin was given in the rear gluteal muscles in doses of 0.5 c.c. of a 1:2 dilution at the same intervals as the drug. This amount corresponded to approximately 166 international units for a 20 Gm. mouse. The antitoxin was used without the drug as a control and also repeated in a small number of animals with the first dose given eighteen hours before infection. The reason for this will be given in the discussion of the results.

After death, animals were examined for abscesses in the kidneys particularly, as well as in other organs; cultures of the heart's blood were taken in those with indefinite or no abscesses and approximately one-half of the others. Survivors were observed for twenty-one days.

RESULTS

In all, 120 mice were used, of which 110 were selected in representative groups satisfactory for statistical purposes. Animals which had died accidentally due to trauma from the peroral injections were discarded.

In the entire group of animals studied, the mean survival time of controls was 6.7 days, whereas in the groups treated with sulfathiazole only and with sulfathiazole and serum, the mean survival times were 13.0 and 12.7 days, respectively. The survival time of animals which received serum only was 6.5 days; this is practically identical with that of the control animals. These figures were subjected to statistical analysis and are recorded in Table II. In a small representative series selected to indicate these factors more clearly, it is noted that one-third of the mice treated with drug only or with drug and serum survived (Table I). Life was definitely prolonged and abscesses noted in only two of those which succumbed. By contrast there were no survivors in the controls or in the serum-treated animals, and abscesses were almost invariably found.

TABLE II

CHEMOTHERAPY AND CHEMOSEROTHERAPY IN EXPERIMENTAL STAPHYLOCOCCUS AUREUS INFECTION: STATISTICAL ANALYSIS OF ENTIRE GROUP

TREATMENT	NO. OF MICE	MEAN SURVIVAL TIME (DAYS)	σ*	STANDARD ERROR OF MEAN
None (control)	30	6.7	2.24	.40
Sulfathiazole	30	13.0	6.7	1.2
Sulfathiazole and serum	30	12.7	6.7	1.2
Serum only	30	6.5	3.82	.84

*σ = Standard Deviation.

DISCUSSION

In common with other observers we found that sulfathiazole prolonged the life of mice infected with *Staphylococcus aureus* and prevented the occurrence of abscesses in the majority of such animals. The properties of this drug (as well as the more recently developed sulfadiazine) are all the more notable, since the massive experimental infection is in direct contrast not only to clinical infection but also to chemotherapeutic experiments upon pneumococci and streptococci. In the latter instance small numbers of virulent bacteria are used, so that quantitative mass relations between the chemical and the bacteria are in favor of the former with resultant sharp readings. The disappointing results with the combination of drug plus serum and with serum only require further elucidation. The potency of the commercial serum is assayed for antihemolytic action, and its dosage is standardized against the international antitoxin unit established by the League of Nations. It contains ample amounts of neutralizing bodies for alpha exotoxin, which is the important toxin in human infections; there is also a fair amount of beta-neutralizing antihemolysin. The latter hemolysin is the hot-cold hemolysin reported by most authors as

incapable of hemolyzing rabbit cells. Roy,[6] however, reports a hot-cold lysin which hemolyzes human as well as rabbit cells despite its resemblance to the beta toxin of Glenny and Stevens.[7] The serum also contains antibacterial bodies which should render it particularly useful in experimental animal infection. Under such conditions bacteremia is a more important issue than toxemia. The time of administration is a factor to be considered, since Farrell and Kitching[8] found that the highest level of absorption in mice did not occur until twelve hours after intraperitoneal injection and eighteen hours after subcutaneous injection. As indicated above, this interval was tested in addition to the method used in the main part of the experiment without materially influencing the results. In addition, such preliminary or prophylactic use of serum bears no resemblance to clinical conditions. We believe that such negative results do not necessarily invalidate the use of sera in clinical infection in which toxemia, longer time element, and reinforcement of natural immunity may tilt the scale in the direction of recovery. A suggestive factor in favor of the serum was noted, though it does not appear in the tables. Occasionally mice treated with drug and serum lived on for several days to a week after cessation of treatment, whereas those treated with drug only succumbed within a day or two after treatment was discontinued. One can speculate that the longer retention of serum created an increment of passive immunity in those mice which were not quickly overwhelmed by the infection.

SUMMARY

1. Sulfathiazole administered perorally prolonged the life of mice infected with *Staphylococcus aureus* and prevented the occurrence of abscesses in the majority of such animals.

2. The mean survival time of animals treated with sulfathiazole was 13.0 days. The mean survival time of those treated with sulfathiazole and staphylococcus antitoxin was 12.7 days. The mean survival time of those treated with serum only was 6.5 days which was practically the same as control animals which received no treatment. The results were further analyzed by statistical methods.

3. Animal experiments with staphylococcic infections do not entirely parallel clinical experience. In the latter certain factors which are discussed indicate that staphylococcic antitoxin may reinforce the immune processes of the host.

REFERENCES

1. Spink, W. W., Hansen, A. E., and Paine, J. R.: Staphylococcic Bacteremia; Treatment With Sulfapyridine and Sulfathiazole, Arch. Int. Med. 67: 25, 1941.
2. Skinner, O., and Keefer, C. S.: Significance of Bacteremia Caused by Staphylococcus Aureus, Arch. Int. Med. 68: 851, 1941.
3. Hoyt, W. A., Davis, A. E., and Van Buren, G.: Acute Hematogenous Staphylococcic Osteomyelitis, J. A. M. A. 117: 2043, 1941.
4. Kolmer, J. A., and Brown, H.: Chemotherapy and Chemoserotherapy of Staphylococcic Infection, Arch. Int. Med. 69: 636, 1942.
5. van Dyke, H. B., Greep, R. O., Rake, G., and McKee, C. M.: Observations on the Toxicology of Sulfathiazole and Sulfapyridine, Proc. Soc. Exper. Biol. & Med. 42: 410, 1939.
6. Roy, T. E.: The Titration of Alpha and Beta Hemolysins in Staphylococcal Toxin, J. Immunology 33: 437, 1937.
7. Glenny, A. T., and Stevens, M. F.: Staphylococcus Toxins and Antitoxins, J. Path. & Bact. 40: 201, 1935.
8. Farrell, L. N., and Kitching, J. S.: Passive Protection of Mice With Immune Serum Against Living Staphylococci, J. Path. & Bact. 50: 439, 1940.

CAROTID SINUS SYNDROME: A REPORT OF THREE ADDITIONAL CASES FROM THE CARDIOLOGICAL SERVICE, U. S. V. A. FACILITY, COLUMBIA, SOUTH CAROLINA

S. L. Zimmerman, M.D., Columbia, S. C.

Hering in 1923 conclusively demonstrated that reflex slowing of the heart might be brought about by carotid sinus pressure. Ample confirmation has followed. Particularly elucidative in this respect was the work of Heymans.[1] It was only in recent years, however, that these findings were made applicable to man. Weiss and Baker[2] in 1933 reported fifteen cases of an abnormally sensitive carotid sinus resulting in attacks of syncope and convulsions. Ferris, Capps, and Weiss[3] in 1935 reported an additional thirty-two cases. Since that time numerous similar cases have been reported and now the entity is universally recognized, but, unfortunately, the role it may play in the causation of syncope and unconsciousness is not always borne in mind. The purpose of this paper is to report three additional cases, which were not diagnosed until the patients were hospitalized.

GENERAL CONSIDERATIONS

It is generally recognized that the reflex arc lies within the autonomic nervous system. The afferent pathways lie chiefly in the intercarotid, the glossopharyngeal, and the hypoglossal nerves; in addition, the vagus and cervical sympathetic chains are also involved. Bucy[4] has beautifully recorded the effects of intracranial section of the glossopharyngeal nerve on the blood pressure. In each instance of his four reported cases, a marked rise in blood pressure resulted from such section, and this rise persisted for several days.

The motor pathways are widespread and variable. This factor alone probably contributes largely to the varied clinical responses. Thus in man, the vagus nerve, the vasomotor depressor nerves, and the central motor pathways may be called into play, individually or collectively, and the resultant clinical entity will depend upon the predominance of any particular pathway. Thus, the three types of syndromes recognized have been a vagal type, characterized by cardiac slowing and asystole, followed by syncope and a convulsion; the depressor type, marked by a severe and often alarming fall in blood pressure; and the central type, in which no observable changes in blood pressure or heart rate take place to explain the syncope or convulsion. Commonly, the entity occurs in a mixed form and then it is most usual to classify it according to the most predominant finding. The pure depressor type is probably the least common, Weiss[6] having observed it in pure form in only two cases.

Signs and symptoms other than syncope are not uncommon. Abdominal manifestations are frequently seen, presumably due to a widespread autonomic discharge of the efferent limb of the reflex arc.[5] In order of frequency, fainting, dizziness and weakness, hyperpnea, pallor, bradycardia and asystole, numbing,

Received for publication, February 12, 1943.

Published with the consent of the Medical Director, Veterans' Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

ness and tingling of the extremities, convulsions, drowsiness, epigastric distress, lacrimation, cough, amnesia, and palpitation are listed by Weiss.[6]

Local disease of the carotid sinus has been reported in the majority of cases, and as a group, the incidence of cardiovascular abnormalities was rather high in the reported cases. The average age lies in the fifth decade, but the youngest case reported by Weiss[6] was 13, and the oldest 74. The three reported cases here lie in the fifth decade.

It is a well-known fact that digitalis sensitizes the carotid sinus reflex, and it should be used with caution in elderly patients who complain of spells of weakness or dizziness. A practical point which might be remembered is the danger of its use in combination with anesthesia and manipulation of the neck in the operating room.

Stimulation of the carotid sinus is not wholly without danger. It is not wise to stimulate both at the same time. Marmor and Saperstein[7] reported a case of bilateral thrombosis of anterior cerebral artery following stimulation of a hyperactive carotid sinus, and Galdston[8] et al. reported two cases of thrombosis of the common, internal, and external carotid arteries, associated with hyperactive carotid sinus reflexes of the other side.

The treatment usually starts off as a medical problem, unless the attacks are so frequent and incapacitating that such treatment is not feasible. The general trend is an attempt at correcting all associated abnormalities wherever possible. Constricting collars should be avoided. Emotional upsets, worry, and fatigue should be minimized if at all possible. Reassurance of the patient with reference to epilepsy and organic heart disease is advisable. Specifically, the vagal type often responds to atropine sulfate, $\frac{1}{150}$ grain three to four times daily, orally; this will often prevent spontaneous attacks. Ephedrine, adrenalin, benzedrine, and paredrine may be used in conjunction, in an effort to irritate the ventricle, a state which is conducive to ventricular escape beats upon the occurrence of the usual sinoauricular block. The dose of any of the above drugs should be the minimum necessary to bring about the desired effect.

The depressor type may often be relieved with any of the sympathomimetic drugs, the longer acting ones being the more desirable. Ephedrine sulfate in doses of $\frac{3}{8}$ grain, three to four times daily, has been satisfactory. Benzedrine is probably longer acting and tolerated better by some. The dose ranges between 10 to 20 mg. three times daily. It is said that the side effects are less.

The cerebral type offers a more difficult problem. Here, prime attention must be paid to the associated pathologic condition. Any deficiencies must be treated along generally acceptable lines. Often surgical denervation offers the best hope in these cases. In any case, however, surgery may have to be resorted to if the medical therapy fails. The results have been good as a whole. Recurrences have been few, and an interesting point in this respect has been brought out by Weiss[6]; namely, that those patients in whom a severe, widespread neurosis, associated with a low basal metabolic rate is present, fare poorly, and such condition is considered by him a contraindication to surgical intervention.

CASE REPORTS

CASE 1.—Mr. T. A. B., Reg. No. 28266, a 58-year-old white male, civil engineer by occupation, was admitted for the first time to the United States Veterans' Administration

Facility at Columbia, South Carolina, February 26, 1942, with an admitting diagnosis of "seizures, type undetermined." The family history was irrelevant. There was no history of epilepsy or other neuropsychiatric disorders in the family. Mother died at the age of 70 from "old age" and pneumonia. Father died at the age of 85. One brother died at the age of 35 of pneumonia.

The past history included the usual childhood diseases. He had typhoid fever at the age of 15, influenza in 1918, was hospitalized in 1932 for "arthritis." Venereal disease was denied.

He was seen by me February 27, 1942, after he had been referred for cardiac consultation by the neuropsychiatric service. The history obtained was as follows: There was no history suggestive of a diminished cardiac reserve, the patient denying exertional dyspnea, dependent edema, paroxysmal nocturnal dyspnea, and precordial or retrosternal pain related to exertion. He was not a known hypertensive. The first attack of syncope occurred in December, 1941, at which time he attempted to switch off the radio while in bed. This necessitated a movement of his head to the right, and it was followed immediately by an unconscious state, during which, his wife stated, both hands were drawn upward toward the shoulders. There was no convulsion, nor was there any incontinence, and he did not bite his tongue. The next attack occurred at 10:30 A.M., February 26, 1942, while attempting to put his right arm in the torn sleeve of his overcoat. That was all he remembered. He was observed to have fallen to the ground, injuring his face and left elbow. The right side of his tongue was cut.

Physical examination at the time of admission revealed a well-developed, well-nourished white male, weighing 158 pounds, 73 inches tall. There were bruises and abrasions of his face and elbow. The lungs were negative. Blood pressure was 128/80, the rhythm regular, and no cardiac enlargement was noted. There were no thrills or murmurs. Arcus senilis was present, and there was peripheral arteriosclerosis. Because of the suggestive history, a carotid sinus syndrome was suspected by the cardiologist, and, on February 27, 1942, a preliminary examination revealed the pulse to be 72, blood pressure 128/80, and the rhythm regular. While in the sitting position, stimulation of the right carotid sinus made the patient dizzy, and he keeled over. The blood pressure fell to 98/60. With this syndrome as the probable diagnosis, other possible etiological factors were investigated by the cardiological service.

The retinal vessels were within normal limits and there was no evidence of increased intracranial pressure. The discs were clear. The electrocardiogram revealed a regular sinus rhythm and a rate of 75. All QRS complexes were upright. The T waves were normal, and there was no significant ST deviation. The conduction was normal throughout. The interpretation was "within normal limits." X-ray of the skull revealed no abnormalities. X-ray of the chest revealed a thickening of the pleura and some coarse peribronchial thickening in the mid-portion and bases of both lung fields. The aortic, cardiac, and diaphragmatic shadows were normal. The red blood count was recorded as 3.69 million with 67.5 per cent hemoglobin on February 23, 1942, and 5.04 million with 73 per cent hemoglobin on March 3, 1942. The smear was not remarkable. The white blood count was reported within normal limits. The sedimentation index was 23 mm. per hour (Westergren). The urine revealed a specific gravity of 1.020 and was entirely normal. The Wassermann and Kahn tests were negative. Spinal tap was performed March 4, 1942. The fluid was clear, the pressure normal. The globulin test was negative; there were 2 cells per c.mm.; the dextrose was normal and the Wassermann reaction was negative. The Lange colloidal gold test was negative. Three basal metabolic rates were obtained, with readings of minus 13 per cent, minus 21 per cent, and minus 25 per cent reported.

On March 2, 1942, the syndrome was again tested. Pressure on the right carotid sinus resulted in syncope. The right side of the face was pulled upward and the patient lost all contact with his surroundings and fell over to the left. The blood pressure fell from 110/80 to zero? The rhythm remained regular and did not slow.

On March 3, he was presented to the Clinical Pathological Conference, Dr. J. Heyward Gibbes of Columbia, presiding. Following stimulation of the right carotid sinus, the blood pressure fell from a level of 140/90 to 50/30, and the patient "passed out" completely. There was no change in pulse rate or rhythm. When stimulation was stopped, the

patient remained confused for a few moments and then returned to his normal state. Again the face was drawn upward on the right and he fell to the left. Pressure on the left carotid sinus failed to result in any abnormal reaction. He was placed on ephedrine sulfate, ¾ grain, twice a day, and given one grain of thyroid daily, receiving no other medication. He had no further spontaneous attacks while hospitalized. Subsequent attempts at causing syncope failed entirely. The patient gained nine pounds in weight and was discharged March 23, 1942, with instructions to continue the intake of ephedrine sulfate. The final diagnoses were:

1. Carotid Sinus Syndrome, Right, with Syncope, Depressor Type.
2. Arteriosclerosis, Radial and Brachial Vessels.

Follow-up: He again reported to me and stated that he suffered with a few more similar attacks, but admitted that he had not continued the ephedrine.

CASE 2.—Mr. W. L. S., Reg. No. 29612, a 46-year-old white male, was admitted for the first time to the United States Veterans' Administration at Columbia, S. C., on July 13th for the treatment of an upper respiratory infection. The family history was noncontributory, except that one sister had died at the age of 44 of Bright's Disease. He had the "usual" childhood diseases, and the remainder of the past history was irrelevant. Syphilis was denied. He used no drugs, nor did he use excessive amounts of alcohol.

The history revealed that the patient contracted an upper respiratory infection resulting in hoarseness and cough which began three weeks prior to admission. During these paroxysms of cough he became unconscious, having had nine such attacks in the preceding three weeks. Two such attacks occurred during the first two weeks of admission.

Routine admission examination revealed a blood pressure of 146/76 and some arteriosclerosis of the radial vessels. A special eye, ear, nose, and throat examination by the consultant revealed an acute catarrhal pharyngitis and laryngitis. He was seen by the neuropsychiatrist, who found nothing of relevance.

Blood and urinary work-up were not remarkable. The Wassermann and Kahn tests were negative. Spinal fluid was normal and the pressure was not increased. X-ray of the skull revealed three unexplained areas of rarefaction in the frontal region. X-ray of the chest was negative. Electrocardiogram revealed left axis deviation, some slurring of the QRS complexes and low T waves in all leads. No conduction defect was noted. The rhythm was normal sinus.

He was seen in consultation by the Cardiological Service, at which time the following was noted: He had enjoyed good health and had no diseases of relevance until eight weeks prior to admission, at which time he "caught a cold" and suffered severe paroxysms of coughing. A tentative diagnosis of whooping cough was made by his local physician. He was not a known hypertensive, was never a congestive failure, and there was no history of coronary insufficiency. The first attack of syncope followed a coughing paroxysm and occurred while the patient was in a sitting position. He lost consciousness (he was told this) for a few minutes. There was no generalized convulsion, no loss of sphincteric control, and he did not bite his tongue. He aroused in a few moments. Eight or nine similar attacks followed, and each was brought on by a short coughing paroxysm. No voluntary movement of the head or neck could precipitate an attack.

The relevant findings in the cardiovascular examination revealed a normal-sized heart with a soft systolic blow over the aortic area. A-2 and P-2 were approximately equal. The pulse was 72 and the blood pressure 110/70 in the sitting position. The radial and brachial vessels were soft and compressible. Visualization of the fundi revealed mild tortuosity of the vessels. There was no evidence of failure.

On August 11, 1942, stimulation of the right carotid sinus caused a typical syncopal attack. There was a complete asystole; no heart sounds at all were heard, and the blood pressure fell to zero. With this definite clue, the entire procedure was repeated and a simultaneous first-lead electrocardiogram was obtained. With the patient in a sitting position, stimulation of the left carotid sinus for about thirty seconds caused the patient to lose consciousness. The eyeballs rolled upward and he fell over to the left side. Recovery was rapid following cessation of stimulation. Complete asystole followed a slowing of the heart rate (Fig. 1).

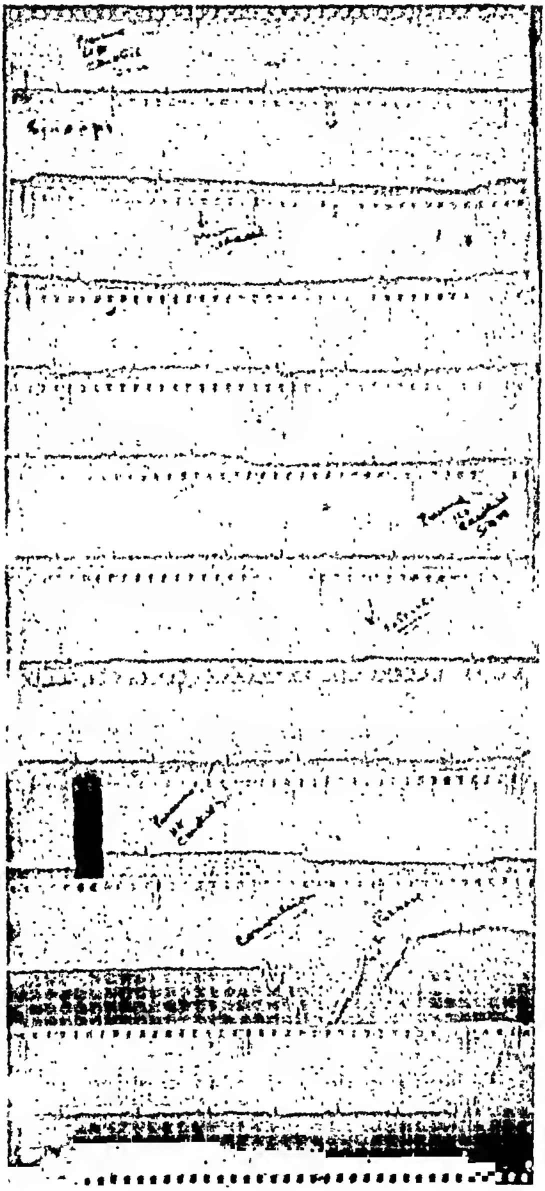


Fig. 1.—Patient W. S. Continuous tracing, Lead I, showing the effect of carotid sinus pressure. At the top of this tracing three complexes are noted, and at the indicated point, pressure was applied on the left carotid sinus. Patient fainted, and the pressure was not applied steadily and constantly. Note that following the asystole there are four contractions recorded, originating in the S-A node. At the arrow the pressure was released and cardiac activity was resumed. At first it was slow and later at a more rapid rate. Pressure on the right carotid sinus was applied, as indicated, and again there is a long pause, followed by an occasional contraction. Pressure was released at the point indicated and then reapplied on the right carotid sinus. This was followed by a complete asystole and syncope and finally a convulsion, at which point the pressure was again released. It is noted that there are no nodal or ventricular escapes.

A short while later the right carotid sinus was stimulated, and pressure for about thirty to forty seconds resulted in loss of consciousness and a short clonic convulsion, which lasted as long as stimulation was continued and ceased soon after the cessation of stimulation of the right carotid sinus. The blood pressure fell, secondarily to the asystole.

The diagnosis was: carotid sinus syndrome, bilateral, with syncope, of the vagal type.

The patient was placed on atropine sulfate, 1/150 grain three times a day, and was discharged soon thereafter, having been advised to continue this medication. He has not been heard of since his discharge on September 19, 1942. Whether or not the upper respiratory infection was the sensitizing factor and the cough the trigger mechanism is unknown. Certainly it appears likely that such was the case.

CASE 3.—Mr. L. S., Reg. No. 29796, a 52-year-old white male, was admitted for the first time to the United States Veterans' Hospital, Columbia, S. C., July 29, 1942, complaining of fainting spells of three years' duration. The family history was noncontributory. He had the "usual childhood diseases," typhoid fever in 1912, and denied any venereal infection.

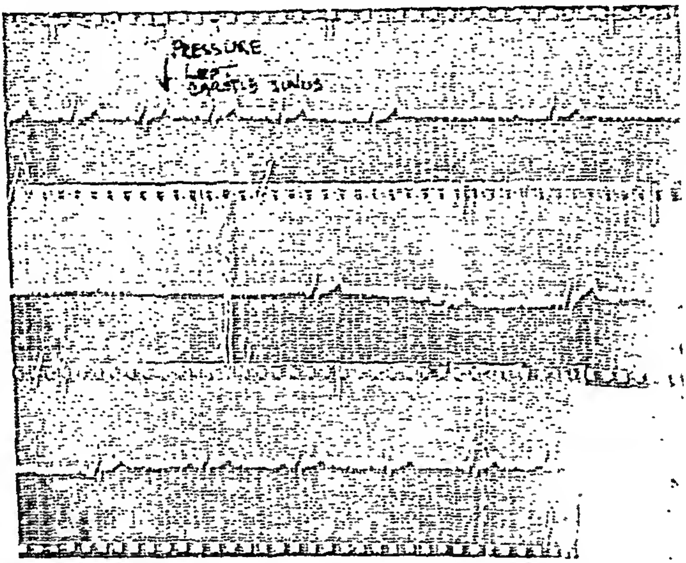


Fig. 2.—Patient L. S. Pressure on left carotid sinus, at the indicated point, resulted in, first, a slowing of the normal sinus rhythm, and then a complete asystole, followed by syncope. The point of release of pressure is not indicated on this tracing, but it is noted that upon cessation of asystole the normal sinus rhythm reappeared, at first slow, and then at a more rapid rate.

The history given to the admitting office revealed that he had "spells," consisting of "shaking and swishing noises in his head," complete blindness, and falling to the ground, for three years prior to admission. He stated that exertion brought on these attacks. Consciousness was regained soon after falling to the ground. He also complained of weakness, stating that he lost 30 pounds during the two months prior to admission. He stated that once the shaking had started, he could prevent the rest of the spell by holding his head still between both hands. The remaining history was irrelevant.

Examination at the time of admission revealed a blood pressure of 120/80. The heart tones were recorded as faint, but there was no enlargement, no murmurs, nor arrhythmia. The remainder of the examination was negative, including a neurological examination.

The blood, urinary work-up, including modified Mosenthal, phenolsulfonphthalein output, nonprotein nitrogen determination, and microscopic urine examinations, were within normal limits. The Wassermann and Kahn tests were negative. Spinal fluid was crystal clear, under normal pressure, and negative in all other respects. The colloidal gold test was negative. X-ray of the skull, lumbosacral spine, and hips was normal. X-ray of the chest was negative. Four-lead electrocardiogram was within normal limits.

He was seen in consultation by the Cardiological Service on August 18, 1942, at which time further history revealed that these attacks could be brought on by unusual movements of the head. The frequency varied, sometimes occurring as often as two or three times per week, but usually not so often. There was no history of bladder or rectal incontinence; he never bit his tongue; and he was never told that he had a generalized convulsion during any of these attacks. At times he said he felt as though a fly were in his left ear. No history was obtained of coronary insufficiency nor of pre-existing hypertension.

Cardiac examination revealed no increase in heart size, regular sinus rhythm, and no thrills or murmurs. The first apical sound was recorded as "poor." A-2 and P-2 were equal. There was no peripheral sclerosis and no evidence of congestive failure. The retinal vessels were normal.

Pressure on the right carotid sinus did not slow the heart rate nor reduce the blood pressure, and resulted in no clinical manifestation of syncope. Very little pressure on the left carotid sinus, of short duration, resulted in complete asystole, followed by syncope. (See Fig. 2.)

A clinical diagnosis of carotid sinus syndrome, left, of the vagal type, with syncope, was made. He was placed on atropine sulfate, by mouth, in doses of $\frac{1}{150}$ grain, for a period of three weeks, had no recurrence, and was discharged on September 2, 1942. He has not been heard from since.

SUMMARY

1. Three cases of carotid sinus syndrome, two of the vagal type and one of the depressor type, are reported. In all, medical treatment afforded some relief. One of the patients with the depressor type had an associated low basal metabolic rate. His response to medication was not reliable, possibly in view of his failure to diligently take the ephedrine. The results with the vagal type cases have been more encouraging.

2. In all three of these reported cases the presence of the syndrome was easily established. In two the history revealed the precipitating factor, to be an abnormal or sudden movement of the head and neck. In the third, the patient learned that he could avoid such an attack by holding his head still between his hands.

3. The syndrome should be borne in mind in all cases of unexplained syncope, especially if associated with abnormal movements of the head and neck.

REFERENCES

1. Heymans, C.: Le sinus carotidien et les autres zones vasosensibles réflexogènes, Paris, 1929, Presses Universitaires de France.
2. Weiss, Soma, and Baker, J. P.: The Carotid Sinus Reflex in Health and Disease: Its Role in the Causation of Fainting and Convulsions, Medicine 12: 297, 1933.
3. Ferris, E. B., Jr., Capps, R. B., and Weiss, Soma: Carotid Sinus Syncope and Its Bearing on the Mechanism of the Unconscious State and Convulsions, Medicine 14: 377, 1935.
4. Bucy, Paul C.: Carotid Sinus Nerve in Man, Arch. Int. Med. 58: 418, 1936.
5. Stern, J. Edward: Abdominal Manifestations of the Hyperactive Carotid Sinus Reflex, J. A. M. A. 110: 1986, 1938.
6. Weiss, Soma, Capps, Richard B., Ferris, Eugene B., Jr., and Munro, Donald: Syncope and Convulsions Due to a Hyperactive Carotid Sinus Reflex, Arch. Int. Med. 58: 407, 1936.
7. Marmor, Judah, and Saperstein, M. R.: Bilateral Thrombosis of Anterior Cerebral Artery Following Stimulation of a Hyperactive Carotid Sinus, J. A. M. A. 117: 1089, 1941.
8. Galdston, Morton, Govons, Sidney, Wortis, S. Bernard, and Taylor, Henry K.: Thrombosis of the Common Internal and External Carotid Arteries, Arch. Int. Med. 67: 1162, 1941.

ADDENDUM

Since the above paper was submitted for publication, three more cases of carotid sinus syndrome of the vagal type were observed.

CASE 1.—The first was noted in a 60-year-old Negro male, a railroad worker by occupation, who had an attack of unconsciousness of unknown cause about six years ago, followed by numerous attacks of giddiness and "swimming sensation in his head." No further attacks of outright syncope occurred and on the outside he was diagnosed as having coronary disease. When seen, he presented marked hypersensitivity of the right carotid sinus, resulting in asystole and syncope. He was a known hypertensive, and electrocardiogram revealed evidences of myocardial disease. Because of the fact he was not admitted for treatment, further observation was impossible.

CASE 2.—The second case noted was in a 53-year-old white male, a textile worker by occupation, who was admitted because of bilateral deafness of the perception type, with degeneration of the right auditory nerve. He had no spontaneous attacks of syncope, and, when routinely examined on the cardiac service, he presented a very definite bilateral carotid sinus syndrome of the vagal type, with asystole and syncope. At present, he is being treated with atropine sulfate, grain $\frac{1}{150}$, orally, three times daily. He has not been observed sufficiently to determine whether or not this will be effective. Aside from some arteriosclerosis of the peripheral and retinal vessels, cardiological work-up was essentially normal. He returned subsequently, having had three spontaneous attacks of syncope. His response to atropine was poor, and he will in all probability come to surgery.

CASE 3.—A 72-year-old white male, a known hypertensive and coronary arteriosclerotic cardiac patient, entered with a history revealing three recent spontaneous syncopal attacks of unknown etiology. The case proved to be one of extremely sensitive left carotid sinus syndrome of the vagal type with asystole and syncope. Atropine and ephedrine, in large doses, have prevented further spontaneous attacks.

THE INFLUENCE OF MUSCULAR WORK AND FATIGUE ON THE STATE OF THE CENTRAL NERVOUS SYSTEM*

ERNST SIMONSON, M.D., NORBERT ENZER, M.D., AND ROY W. BENTON, M.D., MILWAUKEE, WIS.

INTRODUCTION

THE reciprocal relationship between muscular fatigue and fatigue of the central nervous system has theoretical interest and practical application. Most industrial occupations involve muscular work as well as functions of the central nervous system. Hence, the development of general fatigue in industry depends on the influence of both systems on one another. It would seem that knowledge of this relationship could be useful in military affairs, since there are many occupations and situations in which fatigue of the central nervous system contributes to general fatigue arising out of muscular strain or effort. Concerning this question, no experimental material is available, so that Ivy[1] in a recent review states only, "As is well known, heavy muscular exercise influences mental performance because of the tendency to fall asleep." It would be practical to know what amount of muscular work is necessary to produce a depressing effect on the central nervous system, how long the effect lasts, and whether the type of work is an influencing factor. From a theore-

*From The Northwestern Mutual Life Insurance Company and the Research Laboratory of Mt. Sinai Hospital.
Received for publication, February 12, 1943.

tical point of view it would be interesting to investigate the mechanism of the depressing effect. While the depression of the central nervous system by muscular fatigue has been generally accepted as a fact on the basis of subjective experience, little is known of the effects of short, moderate muscular work on the state of the central nervous system; i.e., whether short, moderate muscular work exerts a stimulating effect. The fusion frequency of flicker seems to be a method suitable for the study of the influence of muscular work on the state of the central nervous system. The fusion frequency of flicker is that rate of successive light stimuli necessary to produce an effect equal to continuous illumination. It depends on chronaxie, latent period, and refractory period of the visual system (retina, visual pathways, visual centers); therefore, it indicates the excitability of the visual system, and perhaps, to a certain degree, the excitability of the whole central nervous system. It has been repeatedly demonstrated that general fatigue develops first in the sensory centers.[2] The fusion frequency of flicker is depressed in fatigue of the central nervous system,[3, 4] in pathologic fatigue of patients with hypothyroidism,[5] or circulatory insufficiency;[6] on the other hand, the fusion frequency of flicker increased parallel to the relief of fatigue in patients with hypogonadism after treatment with testosterone,[7] and in normal subjects after benzedrine[8] and pervitin.[9] The fusion frequency of flicker can be measured with great accuracy, and cannot be influenced by effort or by training. The effect of muscular work on the central nervous system probably depends on the physical condition and sensitivity or resistance of the central nervous system of the individual subject. Therefore, equal effects of a given standard type of work cannot be expected to result in different people. Short, moderate exercise might produce a depression of the excitability of the central nervous system in a weak subject, while a rather strenuous muscular effort might fail to do so in a stronger subject. Hence, it would seem to be more proper to express the results of this study in terms of distribution of deviations in a group of 54 subjects. Each type of work might increase the excitability of the central nervous system in some subjects and depress it in others. But a shift from prevailing stimulating effects in the majority of subjects after short, moderate exercise to prevailing depressing effects after more strenuous exercise may be expected. Four different types of work were investigated:

1. Static work, where fatigue is rather localized.
2. Short, moderate exercise (30 genuflexions), without fatigue.
3. Pulley, for three minutes or until fatigue.
4. Running until fatigue.

Although the performance of these different types of work cannot be compared on a quantitative basis, it is clear that the effort increases from Type 1 to Type 4.

METHOD

The fusion frequency of flicker was measured under standard conditions of surrounding illumination, illumination of the test patch, visual angle, light-dark ratio, and exposure. Direct current was used for the light source. Details of method and procedure are described in an earlier publication.[3] The

fusion frequency of flicker was measured before the exercise and ½, 1, 2, 3, etc., minutes after the exercise. The fusion frequency was also measured during the static exercise, where the subject remained in the same sitting position as used for the determination of the fusion frequency. In the other types of exercise, it was not possible to do so.

Types of work.—(a) Static exercise: The subject had to hold a load of 8 pounds with the right arm straight in horizontal position until completely fatigued.

(b) 30 genuflexions were performed within one minute according to the rhythm of a metronome. This exercise did not produce fatigue, so that the subjects would have been able to continue.

(c) Horizontal pull with both arms of a pulley weight of 26 pounds at a rate of 20 per minute for women and 30 per minute for men. The stop signal was given after three minutes, but 16 subjects ceased work earlier because of fatigue.

(d) Running: The subjects were asked to run through a corridor of 33.5 meters' length back and forth until fatigued. Although they were urged to run fast, there were great individual variations of speed (between 1.43 and 4.19 meters per second), total distance (from 201 and 737 meters), and time (from 70 to 300 seconds). There was no inverse ratio between speed and duration, so that the individual differences must be explained by different physical and training condition, and perhaps by different willingness to exhaust themselves. If the subject did not break off the exercise earlier, the stop signal was given after 670 meters (Nos. 6. 17, 19, 20, 29, 33, 39, and 44), in one subject (No. 8) after 737 meters, in order to avoid too great differences in the amount of work performed. One subject (No. 16) broke off the performance after 80 seconds because of side pains, which occurred also in a second investigation after several weeks' interval. Although the running exercise cannot be regarded as standardized, it was for each subject by far the hardest exercise test, compared to the other tests, "a", "b", and "c". Only one exercise test was investigated at a time, so that there was an interval of several weeks between the different exercise tests for each subject. The investigations were performed at the same time (1½ hours after lunch). All results are given as deviation from the initial value before the exercise.

SUBJECTS

The 54 normal subjects (25 men. 29 women) ranged from 18 to 38 years. The average age of the male subjects was 33.3 years, that of the female subjects was 27.4 years. All passed through a medical examination, and none showed any abnormal findings. The height of the group of male subjects varied from 5 feet, 1⅞ inches to 6 feet. 1¾ inches, the weight from 130 to 207½ pounds. The correspondent values for the group of women are: Height 5 feet, 2 inches to 5 feet 9½ inches, and weight from 111 to 141½ pounds. They were employed in sedentary occupations. Only a few (4 men, 1 woman) were in a training condition which could be considered as above the average, i.e., they were participating rather regularly in athletic games or exercises; the others did so only occasionally, at greater intervals, or not at all. In this respect the

TABLE I

EFFECT OF FOUR TYPES OF MUSCULAR WORK ON THE FUSION FREQUENCY OF FLICKER, EXPRESSED IN NUMBER AND PERCENTAGE OF DEVIATIONS RELATED TO THE TOTAL NUMBER OF CASES

1 TYPE OF WORK	2 TOTAL NO. OF CASES	3 ONLY INCREASE	4 INCREASE AND DECREASE	5 ONLY DECREASE	6 ALL INCREASES	7 ALL DECREASES	8 WITHOUT CHANGE
(a) Static Exercise	53	42 = 79.2%	7 = 13.2%	3 = 5.6%	49 = 92.5%	10 = 18.8%	1 = 1.9%
(b) Genuflexions I	54	34 = 63.0%	12 = 22.2%	6 = 11.1%	46 = 85.2%	18 = 33.3%	2 = 3.7%
Genuflexions II	47	31 = 66.0%	10 = 21.3%	5 = 10.6%	41 = 87.3%	15 = 31.9%	1 = 2.1%
(c) Pulley	49	20 = 40.8%	18 = 36.7%	10 = 20.4%	38 = 77.5%	28 = 57.2%	1 = 2.0%
(d) Running	52	2 = 3.8%	11 = 21.2%	39 = 75.0%	13 = 25.0%	50 = 96.2%	

experimental group may be regarded as a fairly good average of normal population.

During the period of investigations several subjects dropped out, so that the total number of subjects was 53 for the static exercise, 52 for the running exercise, and 49 for the pulley exercise. At the end, test "b" (genuflexions) was repeated on 47 subjects. Thus, the experimental material covers about 2,500 determinations of the fusion frequency.

RESULTS

Table I shows the effect of the four types of muscular work on the fusion frequency of flicker, expressed as frequency of positive (stimulation) and negative (depression) deviations, absolute as well as in percentage of the total number of cases (column 2). Column 3 shows the number of cases who responded to exercise with only an increase, column 5 those who responded only with decrease, while column 4 shows the cases where increase as well as decrease were observed during the recovery period. Column 6 shows all cases with increase, i.e., the sum of columns 3 and 4, while column 7 shows all cases with depression, i.e., the sum of columns 4 and 5. Only in five experiments, muscular exercise (genuflexions, pulley exercise) failed to produce a definite response; in all other experiments the deviations exceeded the experimental error. Table I shows that any type of exercise may produce an increase of the fusion frequency of flicker in some subjects and a depression in others, but there was a pronounced shift of their distribution. The number of cases who responded only with increase (column 3) decreased from 79.2 per cent in static exercise to 63.0 and 66.0 per cent in genuflexions, to 40.8 per cent in the pulley exercise, and to 3.8 per cent in the hardest type of exercise (running). Accordingly, the number of cases who responded only with depression (column 5) was lowest in the static exercise (5.6 per cent) and highest after running (75 per cent). The same inverse relationship can also be seen in columns 6 and 7. While increase after static exercise and genuflexions occurs in 85 to 90 per cent of all cases, a depression is observed in 96.0 per cent after running. These results show that muscular exercise may increase as well as depress the excitability of the central nervous system; the factors responsible for the stimulating effect prevail in short, moderate exercise, while the depressing factors prevail in hard exercise. In intermediate types of exercise, such as the pulley exercise, 36.7 per cent show increase as well as decrease. The error of the percentage of increases (column 6) and decreases (column 7) was calculated according to the formula $\pm\sqrt{\frac{P_1\% \cdot P_2\%}{n}}$ where P_2 per cent $= 100 - P_1$ per cent (Poll[11]).

For the static exercise, the error of the percentage of all increases was ±3.618; that of the percentage of all decreases was ±5.367. The corresponding values for Genuflexions I were ±4.832 and ±6.404, respectively; for Genuflexions II, ±4.873 and ±6.783, respectively; for the pulley exercise, ±5.965 and ±7.069, respectively; for the running exercise, ±6.005 and ±2.699, respectively. The limit of statistical reliability is regarded to be three times these values. This means that the prevailing increase of the fusion frequency after static exercise

TABLE II

LIMITS, AVERAGE, AND DISTRIBUTION OF MAXIMUM DEVIATIONS OF THE FUSION FREQUENCY OF FLICKER, IN PERCENTAGE OF THE TOTAL NUMBER OF OBSERVATIONS, AFTER FOUR TYPES OF MUSCULAR PERFORMANCE

TYPE OF WORK	1	2	3	DISTRIBUTION 4											
	MAX. DECR.	MAX. INCR.	AVER.	-10 TO -8.1	-8 TO -6.1	-6 TO -4.1	-4 TO -2.1	-2 TO -0.1	+2 TO +0.1	+4 TO +2.1	+6 TO +4.1	+8 TO +6.1	+10 TO +8.1	+12 TO +10.1	OVER +12.1
(a) Static Exercise	-4.4	+13.2	+3.84			3.4	6.8	6.8	13.5	28.9	25.4	8.5		5.1	1.7
(b) Genuflexions I	-5.6	+14.0	+2.46			3.1	14.1	10.9	12.5	28.1	18.8	9.4	1.6		1.6
Genuflexions II	-5.8	+13.2	+2.97			3.6	10.9	12.7	14.5	36.4	7.3	5.5	5.5		3.6
(c) Pulley	-8.8	+20.8	+0.97	1.5	1.5	7.5	19.4	13.4	17.9	23.9	5.9	5.9	1.5		1.5
(d) Running	-9.6	+ 8.0	-3.77	3.2	14.5	30.6	24.6	6.4	6.4	6.4	6.4	1.6			

and genuflexions and the prevailing decrease after running are statistically significant. On the other hand, the prevailing of the percentage of increases after the pulley exercise is only a tendency without statistical significance. Significant are also the high percentage of increases in types "a" and "b," compared to the low percentage in type "d"; and the high percentage of decreases in type "d," compared to the lower percentage in types "a" and "b." It is interesting that the amount of work producing a depression of the fusion frequency is rather small; in three subjects the speed of running was slow (subjects 1, 12, and 49), or the distance was small (subjects 3, 12, 21, 22, and 49); nevertheless, a distinct depression was observed. Only a rather short, moderate type of work produces increase of fusion frequency in the majority of *normal* subjects. There was no direct relationship between the frequency or the magnitude of the increase in types "a" and "b" and the training condition. For instance, genuflexions failed to produce any distinct deviation in one of the best trained subjects (No. 8). It is possible that the "moderate" type of exercise was too slight for this subject to provoke any response of the central nervous system.

Table II shows the magnitude of the deviation of fusion frequency after muscular work. Only the maximum deviation is considered. Column 1 shows the maximum depression of the whole group; column 2 shows the maximum increase. The lowest depression is –4.4 after static exercise and increases to –9.6 after running. Occasionally also, moderate types of work may produce marked depression. The upper limit of increase (column 2) is smallest after running (+8.0). The distribution (4) is given in intervals of deviation from –10 to –8.1, from –8.0 to –6.1, etc., as percentage of the total number of observations. The distribution of the values in intervals of distinct, but not extreme, responses, such as –4 and –6, and +4, reflect the tendency much better than the stray values of extreme limits (columns 1 and 2). The percentage of observed depressions in the intervals –4 and –6 increases from type "a" to type "d," so that for type "d" depressions ranging between –2.1 and –4.0 are about four times as frequent as for type "a," and this is even more manifest for the interval between –4.1 to –6.0 deviation. Type "b" (genuflexions) was repeated after an interval of three months. For the second investigation only 47 subjects were available. The limits, averages, and distribution of increase and depression are similar in the first (Genuflexions I) and second (Genuflexions II) investigation. This shows that the distribution of values is rather constant and characterstic for the given type of exercise.

Table III shows the duration of deviations of the *fusion* frequency, subdivided into two groups with durations of less or more than eight minutes. The time of eight minutes is arbitrary. The duration of the increase exceeds eight minutes in ten to fourteen cases in types "b" and "c," while five cases after static exercise and only one case after running show a duration of increase exceeding eight minutes. The lower incidence of longer lasting increases after static exercise may be explained by the comparatively small amount of work performed in this type of exercise. Also, the duration of decrease is less than eight minutes in all cases after static exercise, and in 28 of 31 observations after Genuflexions I and II. There are almost as many cases with depressions ex-

ceeding eight minutes as those less than eight minutes after pulley exercise, while the depression lasts longer than eight minutes in the majority of cases after running. In several cases, the values were still distinctly depressed sixteen and nineteen minutes after the exercise. A short, hard exercise may depress the excitability of the central nervous system for a rather prolonged time.

No relationship was found between speed, time, and distance in running and the magnitude of deviations. The deviations apparently depend at least as much on individual constitutional factors as on the amount of work performed.

TABLE III

DURATION OF DEVIATIONS OF FUSION FREQUENCY OF FLICKER AFTER MUSCULAR WORK

TYPE OF WORK	DURATION OF INCREASE: LESS THAN EIGHT MINUTES	DURATION OF INCREASE: MORE THAN EIGHT MINUTES	DURATION OF DECREASE: LESS THAN EIGHT MINUTES	DURATION OF DECREASE: MORE THAN EIGHT MINUTES
(a) Static Exercise	43	5	9	–
(b) Genuflexions I	35	11	16	2
Genuflexions II	27	14	12	1
(c) Pulley	28	10	15	13
(d) Running	11	1	15	35

Fig. 1 shows two typical responses for each type of exercise: The short, but pronounced, increase during and after static exercise, the prolonged depression after running, and the wavelike reaction after pulley exercise. The response after running is also wavelike; however, with the level below the resting value, while the waves oscillate around the resting value after pulley exercise. Wavelike reactions also were observed on an elevated level after genuflexions, although less frequent.* It appears that stimulating as well as depressing factors are present during the whole period of recovery after exercise, reaching their maximum at different times.

Our material (25 men, 29 women) allows the comparison of performance as well as of response of the fusion frequency between men and women. The comparison of performance between men and women has some interest because the material on this question is scanty. A difference of the average between the group of men and the group of women was regarded to be significant when exceeding the value of $2\sqrt{SE_1^2 + SE_2^2}$. The average duration of static performance in 25 men was 53 seconds, that of 28 women only 27 seconds. The difference (26 seconds) was highly significant, the value of the expression being only 6.672.

The pulley exercise was stopped after three minutes, so that no statistical comparison of endurance is possible. The male subjects worked at a rate of 30 per minute, the women at the rate of 20 per minute, so that the performance of women was only ⅔ that of men. There is some evidence, however, that the endurance of women was inferior also in this type of work. Out of 25 men, only three stopped working before three minutes because of fatigue, while 13 out of 24 women did so, although they worked at a slower rate.

In regard to running exercise, the performance of women was inferior in all respects (distance, speed, duration). The subjects were instructed to run

*Allen and Schwartz[10] observed wavelike responses of the fusion frequency of flicker after stimulation of the sense of taste, smell, and after acoustical stimulation.

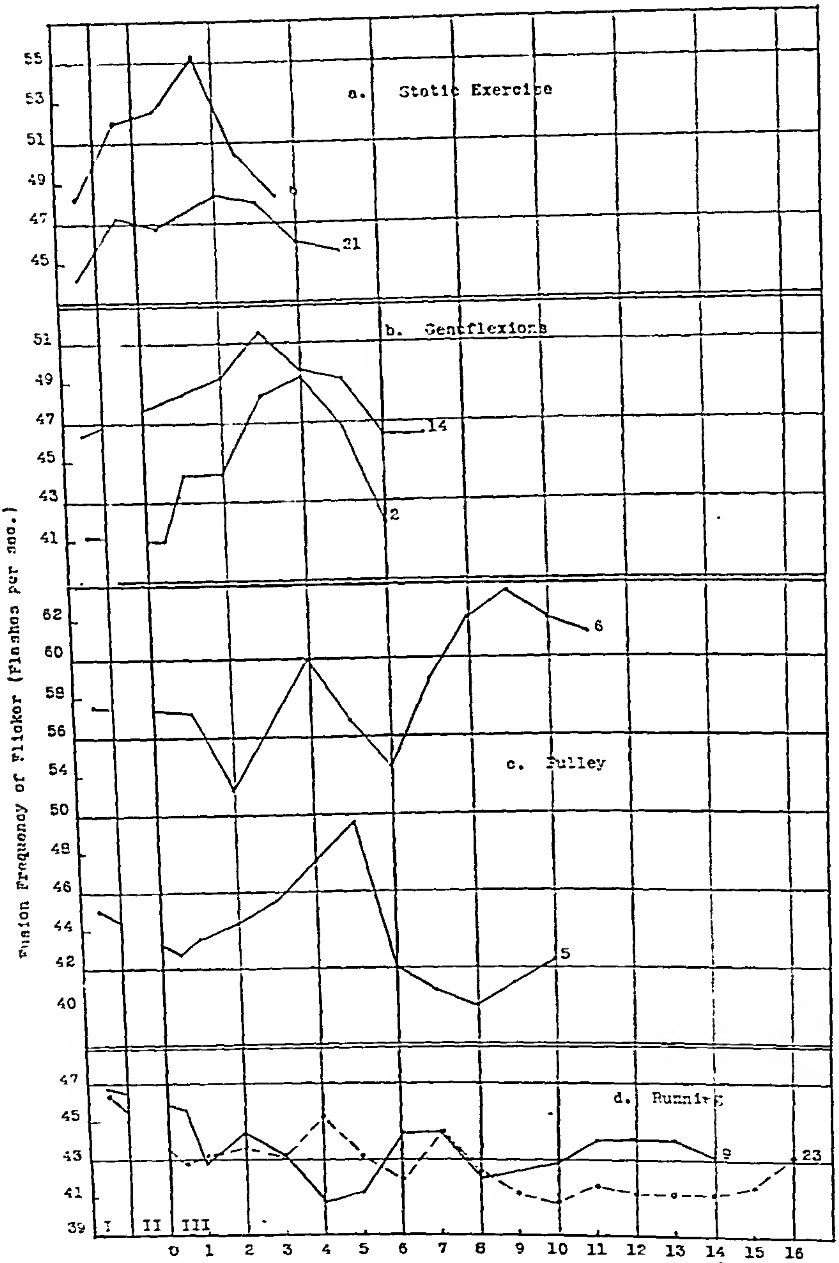


Fig. 1.—Typical effect of four types of muscular work with increasing effort (*a*, static exercise; *b*, genuflexions; *c*, pulley exercise; *d*, running) on the fusion frequency of flicker (flashes per second). "I" means the initial fusion frequency before the exercise; "II" means the period of exercise; during this period the fusion frequency was measured only in type a; "III" means the recovery period after the exercise. The subjects, from whom the curves are taken, are indicated by their numbers. The typical response after short, moderate exercise (types a and b) is an increase of the fusion frequency, while hard exercise (type d) produces prolonged depression. The fusion frequency is still below the initial level, fourteen and sixteen minutes after the exercise. Moderately heavy work (type c) produces increase as well as drop of the fusion frequency.

TABLE IV

INFLUENCE OF SEX ON THE MAXIMUM DEVIATION OF THE FUSION FREQUENCY OF FLICKER AFTER 30 GENUFLEXIONS

1 Exercise	Sex	2 No.	3 Only increase	4 Increase and decrease	5 Only decrease	6 All increases	7 All decreases	8 Without change	9 Average	10 Diff.	$2\sqrt{SE_m^2 + SE_f^2}$
Genuflexions I	Men	25	19 = 76.0%	3 = 12.0%	2 = 8.0 %	22 = 88.0%	5 = 20.0%	1 = 4.0 %	+3.14		
	Women	29	15 = 51.7%	9 = 31.0%	4 = 13.8 %	24 = 82.7%	13 = 44.8%	1 = 3.4 %	+1.87	1.27	
Genuflexions II	Men	24	19 = 79.1%	3 = 12.5%	2 = 8.35%	22 = 91.6%	5 = 20.8%	0	+3.58		
	Women	23	12 = 52.3%	7 = 30.4%	3 = 13.1 %	19 = 82.7%	10 = 43.5%	1 = 4.35%	+2.33	1.25	
Total	Men	49							3.36		
	Women	52							2.08	1.28	1.399

fast, but the speed was not regulated. The average distance men ran until fatigue was 598 meters; that of women 331 meters. The difference (267 meters) was significant ($2\sqrt{SE_1^2 + SE_2^2} = 66.224$). The average speed of men was 282 cm. per second; that of women, 230 cm.; the difference (52 cm. per second) was significant (value of the expression: 20.72). Also the duration was significantly lower in women. This corroborates results of Metheny et al.,[12] who found the average endurance in running at constant speed on a treadmill half as much in a group of 17 women as in a group of 30 men.

In all types of exercise, "a," "c," and "d," the performance of women was smaller than that of men. No significant difference of the response of the fusion frequency of flicker could be seen in these types. It must be assumed, therefore, that the smaller amount of exercise elicited the same response in women as the greater amount of exercise did in men. In type "b" (genuflexions) the amount of work was the same for men and women. The average deviation of the fusion frequency of flicker after the exercise is +3.36 in men and +2.08 in women (total of all experiments, Table IV). The difference (+1.28) is somewhat smaller than the value of the expression (1.399), but both values are almost equal. Although the difference is not quite significant, there is an outspoken tendency to a higher increase of the fusion frequency in men than in women. The same tendency is reflected in the distribution of increase and decrease; 76 and 79 per cent of men (Series I and II, respectively) showed only increase (column 3) after the exercise, while only 52 per cent of the women reacted in the same way. Correspondingly, 20 per cent of men and 44 per cent of women showed a decrease of the fusion frequency (column 7).

DISCUSSION

There are four possible factors responsible for the deviation of the fusion frequency of flicker after muscular exercise.

1. *Circulation.*—The minute volume of blood flow increases during exercise, including the blood flow to the brain. In moderate exercise the oxygen demand of the brain is probably only slightly increased, so that the oxygen supply to the brain exceeds the oxygen demand. This improvement of oxygen supply is likely to increase the fusion frequency of flicker.

2. *Oxygen Debt.*—The huge oxygen debt after hard exercise expresses a general oxygen want including the brain tissue, i.e., the general oxygen demand exceeds the oxygen supply. This deterioration of the oxygen supply is apt to decrease the fusion frequency of flicker.

It is clear that factor "1" will prevail after moderate exercise, factor "2" after hard exercise such as running. This would explain the prevailing increase of fusion frequency after moderate work and the prevailing decrease after hard work.

3. *Radiation of Stimulation.*—A radiation of voluntary impulses from the motor centers to other parts of the central nervous system is generally assumed. This radiation, for instance, is believed to be responsible for the early stimulation of the respiratory center in muscular exercise. The existence of impulse radiation can be shown by the early increase of fusion frequency during static exercise (Fig. 1). The fusion frequency increased twenty seconds after the

onset of static exercise in 83 per cent of all observations. This time is too short to account for any circulatory factor. Radiation of stimulation should be assumed also in the other types of exercise, although it cannot be as readily demonstrated because of the longer duration of exercise and the later time of measurement of the fusion frequency. Although radiation of impulses can be demonstrated only in the early phase of exercise, before the possible interference of circulatory factors, there is no reason to believe that their existence is restricted only to that period. According to Allen and Schwartz's results, reflex stimulation or inhibition of the fusion frequency of flicker might be observed for several minutes after acoustical stimulation.

4. *Radiation of inhibition.*—It seems logical to assume a radiation of inhibition as well as radiation of stimulation from the motor centers. According to Wedenski[13] and Ukhtomski,[14] strong stimuli are likely to produce and spread inhibition to other parts of the nervous system, while moderate stimuli are likely to radiate stimulation. This, too, would explain the distribution of prevailing stimulation after short, moderate exercise and of prevailing depression after running.

The coexistence of nervous and circulatory factors would account for the complicated form of reaction (Fig. 1) after muscular exercise.

In earlier communications we have shown that subjective fatigue in types of work without muscular effort parallels rather closely the depression of fusion frequency of flicker. There is no reason to expect such close relationship between subjective fatigue and drop of fusion frequency after muscular work, because of the interference of muscular fatigue. Static exercise "a" was continued to subjective fatigue; nevertheless, it produced an increase of the fusion frequency of flicker, probably because the fatigue is localized in a rather small part of the central nervous system and because the increase of the oxygen demand is very slight. Certainly, the response of the fusion frequency of flicker does not depend only on the type and amount of exercise, but also on the state and condition of the central nervous system. In other words, the reaction to the same type of work will vary in the same subject when the condition of the central nervous system is altered. We have a chance observation to support this view. In a male subject of 37 years the pulley exercise was investigated two days after he had lost his father. He was in a state of depression and fatigue (due to the loss of sleep during several preceding nights). The exercise depressed the fusion frequency by −5.4 flashes. The test was repeated after two months, when the subject had overcome his depression and was in normal condition. At this time, he responded to the exercise with an increase of +5.2 flashes, which lasted for more than eight minutes.

From a practical point of view, these results indicate that a hard exercise of rather short duration may depress the state of the central nervous system for a comparatively long period of time. This explains the depressed capacity for mental work or other occupations involving nervous functions on the background of physical fatigue. Perhaps, this also accounts for the increased accident rate in fatigue, because of the probable deterioration of alertness. It can be concluded that in occupational work involving both muscular and nervous functions diminution of muscular fatigue will probably diminish

fatigue of the central nervous system. Short, moderate exercise may improve the state of the central nervous system. This would explain the favorable effect of gymnastics during rest pauses observed in several industrial plants. About 10 per cent of normal men and about 20 per cent of women, however, would probably react with depression even after short, moderate exercise. Therefore, a selection for this procedure is advisable. The selection could be made by using the fusion frequency of flicker.

SUMMARY

The reactions of the fusion frequency of flicker of 54 normal subjects after four different types of exercise of increasing severity ("a," static exercise; "b," 30 genuflexions; "c," pulley exercise; "d," running) were studied. Each exercise produced definite deviations of the fusion frequency of flicker. The majority of normal people (about 80 to 90 per cent) respond with an increase of the fusion frequency after static exercise and 30 genuflexions, and with a decrease of the fusion frequency after running. After pulley exercise, about 40 per cent of the subjects respond with both increase and decrease. The magnitude and the duration of the depression correspond to the severity of exercise. These results may explain the depressing effect of physical fatigue after severe muscular exercise, and the stimulating effect of short, moderate exercise on the state of the central nervous system. The endurance of women in static exercise, pulley exercise, and running is significantly lower than that of men, while there is no significant difference of the reaction of the fusion frequency of flicker. The response of the fusion frequency of women after an equal amount of work (30 genuflexions) appears to be inferior to that of men.

REFERENCES

1. Ivy, A. C.: The Physiology of Work, J. A. M. A. 118: 569, 1942.
2. Simonson, E.: Der Heutige Stand der Theorie der Ermuedung, Ergebn. d. Physiol. 37: 299, 1935.
3. Simonson, E., and Enzer, N.: Measurement of the Fusion Frequency of Flicker as a Test for Fatigue of the Central Nervous System, J. Indust. Hyg. & Toxicol. 23: 83, 1941.
4. Lee, R. H.: Critical Fusion Frequency of Flicker, Pub. Health Bull. No. 265, 195, 1941.
5. Enzer, N., Simonson, E., and Blankstein, S. S.: The State of Sensory and Motor Centers in Patients With Hypothyroidism, Ann. Int. Med. 15: 659, 1941.
6. Enzer, N., Simonson, E., and Blankstein, S. S.: Fatigue of Patients With Circulatory Insufficiency, Investigated by Means of the Fusion Frequency of Flicker, Ann. Int. Med. 16: 701, 1942.
7. Simonson, E., Kearns, W. M., and Enzer, N.: *Effect of Oral Administration of Methyltestosterone on Fatigue in Eunochoids and Castrates*, Endocrinology 28: 506, 1941.
8. Simonson, E. Enzer, N., and Blankstein, S. S.: Effect of Amphetamine (Benzedrine) on Fatigue of the Central Nervous System, War Med. 1: 690, 1941.
9. Simonson, E., and Enzer, N.: Effect of Pervitin (Desoxyephedrine) on Fatigue of the Central Nervous System, J. Indust. Hyg. & Toxicol. 24: 205, 1942.
10. Allen, F., and Schwartz, M.: The Effect of Stimulation of the Senses of Vision, Hearing, Taste, and Smell Upon the Sensibility of the Organs of Vision, J. Gen. Physiol. 24: 105, 1940.
11. Poll, H.: Hilfsmittel fuer die Erfolgsstatistik, Klin. Wchnschr. 7: 1777, 1928.
12. Metheny, E., Brouha, L., Johnson, R. E., and Forbes, W. H.: Physiologic Responses of Women and Men to Moderate and Strenuous Exercise, Am. J. Physiol. 137: 318, 1942.
13. Wedenski, N. E.: Die Erregung, Hemmung und Markose, Arch. f. d. ges. Physiol. 100: 1, 1903.
14. Ukhtomski, A.: Excitation, Inhibition, and Fatigue (Russ.), Fiziol. zhurn. 17: 1114, 1934.

THE SIGNIFICANCE OF ERYTHROCYTIC PSEUDOAGGLUTINATION*

ROBERT D. BARNARD, M.D.,† CHICAGO, ILL.

PSEUDOAGGLUTINATION is a term used by Wiener[1] to designate all types of erythrocyte aggregation likely to cause confusion in the clinical determination of blood grouping. Included under this term, he lists rouleaux formation and the aggregation of erythrocytes which may be found in aged whole fluid blood ("autoagglutination").

The same term, pseudoagglutination, was used by Barnard[2] in connection with a certain type of aggregation observed when erythrocytes are acted upon by weak alkali. This type of aggregation appears to be due to a saponification of the cholesterol-lecithitides of the stroma causing the cells to stick together. It appears probable now that the "autoagglutination" of Wiener and the aggregation of rH-positive erythrocytes by the agency of anti-rH factor is of a similar nature, that the phenomenon may be produced by a variety of agents, and that pseudoagglutination, as latterly described, is a precursor to lipolytic hemolysis. For these reasons, it is suggested that the term, pseudoagglutination, be reserved for the specific prehemolytic process to be described, and for the purposes of distinction and that of classification pseudoagglutination may be compared with the other, better-known forms of erythrocytic aggregation; i.e., (1) rouleaux and (2) specific hemagglutination.

1. *Rouleaux*.—This is a physiologic process, having a definite teleologic role in the maintenance of capillary circulation. The mechanism underlying rouleaux is a physical one, as admirably demonstrated by the experiments of Kegerreis.[3] Rouleaux masses are unidimensional, the cell aggregates forming only by apposition of their biconcave surfaces, and the phenomenon, as would be expected from its physiologic nature, is entirely reversible. While this form of aggregation may be seen macroscopically, its designation, as rouleaux, is a microscopic procedure.

2. *Specific Hemagglutination*.—The mechanism of hemagglutination is immunologic. The aggregates are tridimensional, the clumps forming by indiscriminate apposition of groups of cells. Thus in an agglutinated mass, a single cell may be in contact simultaneously with many others, while in rouleaux, no erythrocyte may be in aggregated contact with more than two others. Hemagglutination is a completely irreversible process, and the agglutinated cells are morphologically and structurally altered as evidenced by their susceptibility to hemolytic agents. The designation of an aggregation phenomenon as a true agglutination may be made microscopically, but it is even more characteristic, when complete, on macroscopic examination.

3. *Pseudoagglutination*.—The mechanism appears to be chemical and to consist of a saponification of the cholesterol envelope. Thus, it can be pro-

*From the Department of Therapeutics, Cook County Hospital.

†Supervisor, Blood Bank, Cook County Hospital, 1936 to 39. Present address 1520 Service Unit, Station Hospital, Fort Hayes, Columbus, Ohio.

Received for publication, February 16, 1943.

duced by dilute alkalis or, more rapidly, by toluene. The role of the latter, as a hemolytic agent, is by reaction with the cholesterol-lecithitides of the stroma. (Svedberg and Nichols,[4] 1927; Barnard,[5] 1940.) The cell membrane becomes sticky, and where erythrocytes come into juxtaposition, they tend to adhere. Since the greater proportion of contacts will be at the circumferential surface, pseudoagglutinative aggregates tend to be bidimensional. This is seen best in microscopic preparations with alkali as previously described,[2] and particularly if there are some cotton fibres in the field, the tendency of the cells to adhere to such fibres by their edges is clearly shown. At the same time, changes in the cell morphology under the influence of the hemolytic agent is evident; the cells swell, lose their biconcave shape, and gradually disappear along with liberation of the contained hemoglobin.

TABLE I

	DIFFERENTIATION OF ERYTHROCYTIC AGGREGATION		
	BOULEAUX	PSEUDOAGGLUTINATION	HEMAGGLUTINATION
Mechanism	Physical	Chemical	Immunologic
Aggregates	Unidimensional	Bidimensional	Tridimensional
Reaction	Reversible	Reversible or Irreversible	Irreversible
Change in Cell Morphology	None	Plasmolysis	Crenation
End Result	None	Hemolysis	Hemolysis (in vivo)
Best Determined	Microscopically	Microscopically	Macroscopically

By adjusting the concentration of alkali in which the erythrocytes are immersed, it is possible to demonstrate varying degrees of reversibility for this process. Extremely dilute alkali (1 to 3000 NaCN) will induce a type of aggregation remarkably similar in both microscopic and macroscopic aspects to that produced in rH-positive erythrocyte suspensions by anti-rH sera. Such aggregates may be broken up, after their formation, by vigorous agitation. Again, if such a suspension is allowed to settle in a test tube at room temperature, or if it is centrifuged, all cells are aggregated into a solid mass, but if the treatment be not too prolonged, the cell masses may be broken up by agitation. Since this sequence may be duplicated frequently in reaction mixtures of rH-positive cells and anti-rH sera, the concordance in their behavior between two supposedly diverse agents leads to the belief that, if pseudoagglutination is a precursor to hemolysis, the anti-rH factor is a *hemolysin* rather a true agglutinin. It remains to prove the major premise; the pseudoagglutination is indeed such a precursor, and to this end we have studied the pseudoagglutination produced by dextrose and that appearing spontaneously in aged, stored blood.

THE EFFECT OF DEXTROSE ON ERYTHROCYTIC AGGREGATION

Pendleton[6] reported an agglutinative action of concentrated dextrose or sucrose solutions on human erythrocytes. Such an action, he thought, might explain certain reactions occurring during the intravenous infusion of dextrose solutions. At the Cook County Hospital, we have routinely employed dextrose as a storage medium for blood for transfusion and had definitely demonstrated

that concentrations of dextrose up to 5 per cent (higher than could ever be sustained in vivo) were without any obvious effect on stored blood or its erythrocytes.* We had in fact demonstrated that somewhat higher concentrations of dextrose (12 to 20 per cent) completely inhibited the agglutination of erythrocytes by their own type-specific serum and that this inhibition was reversible in that the cells could be agglutinated when the concentration of dextrose was reduced. The inhibition of the interaction between specific hemagglutinin and hemagglutininogen (for this is what the action of dextrose proved to be) was masked in dextrose concentrations above 20 per cent by the phenomenon that Pendleton described, a phenomenon which has all the characteristics of pseudoagglutination and is invariably followed by hemolysis.

PSEUDOAGGLUTINATION OF ERYTHROCYTES IN AGED, STORED BLOOD

The blood bank has afforded the opportunity for observations on a large number of samples of stored, citrated dextrose-whole blood with regard to their tendency toward pseudoagglutination and hemolysis. While there is a considerable difference between individual samples in the rate at which either of these phenomena make their appearance, there is a distinct correlation between the two in any single specimen. After from one to twenty days of refrigeration, without agitation, the corpuscular layer will be a dark blue-violet color. While the supernatant plasma may show no evidence of hemolysis, it may have been proceeding for some time in the corpuscular layer, and the evidence thereof may have been confined to the corpuscular layer because of the low rate of diffusion of hemoglobin. If the cells in such a sample be suspended by gentle agitation, on their resettlement, free hemoglobin will be evident in the plasma. Such samples show many pseudoagglutinative aggregates.

Since hemolysis is present in these samples and may escape detection, except by allowing an inspection of the plasma after resuspension of the corpuscles, the need for inspection, in this manner, of stored whole bloods, before they are used for transfusion, is obvious.

In the main, samples of blood which showed early hemolysis would likewise show early pseudoagglutination, and this parallelism is carried over to bloods which reacted positively to the serologic tests that are supposed to be diagnostic of syphilis. Naturally these samples were never used for transfusion, even for a known syphilitic prospective recipient (on the theory that although it was not possible to give an individual more syphilis than he already has, he might be given a different kind), and consequently were subject to observation over a long period of time. Curiously enough, in serologically positive blood samples, both pseudoagglutination and hemolysis appeared to be markedly delayed.

It is true that many bloods exhibited gross hemolysis which seems to have occurred spontaneously in the stored samples without showing any pseudoagglutination, and the converse is also true: that samples exhibiting marked pseudoagglutination might show no greater tendency to hemolyze in the stored condition than unaltered bloods. In certain samples, the corpuscles will not mix diffusely with the plasma on agitation, but will swirl around in large tailed,

*It has been noticed, however that even 5% dextrose accelerates or facilitates the appearance of hemolysis in pooled, incompatible bloods.

discoid masses, the bidimensional nature of which is apparent macroscopically. These masses we formerly referred to as "clots" until they were found to be devoid of fibrin. The fibrinogen was still present in undiminished amount in the plasma, and these aggregations showed, microscopically, all the characteristics of pseudoagglutination. Before the nature of this aggregation was appreciated; because the erythrocytes could be separated by continued agitation and because the samples showed little or no hemolysis at this stage, some of these bloods were used for transfusion. The practice was discontinued because of the high incidence of severe hemolytic reactions.

From a practical standpoint, therefore, the presence of pseudoagglutination in a blood sample precludes the use of that sample for transfusion even though hemolysis may be absent. The pseudoagglutinated erythrocyte must be regarded as a potentially hemolyzed one, and the clinical significance of pseudoagglutination is identical to that of specific hemagglutination in incompatable blood.

SUMMARY

The term pseudoagglutination is restricted to a phenomenon observed in erythrocytes which have been acted upon by lipolytic hemolysins. Criteria are set up for its distinction from rouleaux and from specific hemagglutination.

Toluene, high concentrations of dextrose, and anti-rH factor action produce pseudoagglutination, which likewise appears spontaneously in stored whole blood.

Stored blood samples which show positive serologic tests for syphilis appear to be more resistant to spontaneous pseudoagglutination and hemolysis than do serologically negative bloods.

Pseudoagglutination is a precursor to, or a manifestation of, the initial stages of hemolysis by lipolytic agents.

Blood samples which show pseudoagglutination, even without manifest hemolysis, are unsuitable for transfusion.

REFERENCES

1. Wiener, A. S.: Blood Groups and Blood Transfusion, Springfield, Ill., 1935, Charles C Thomas.
2. Barnard, R. D.: Pseudoagglutination of Erythrocytes by Alkali, J. Lab. & Clin. Med. 23: 98, 1937.
3. Kegerreis, R.: Rouleau Formation, J. Lab. & Clin. Med. 22: 581, 1937.
4. Svedberg, T., and Nichols, S. B.: An Oil Turbine Ultracentrifuge, J. Am. Chem. Soc. 49: 2920, 1927.
5. Barnard, R. D.: A Note on the Preparation of Hematoporphyrin, J. Lab. & Clin. Med. 25: 747, 1940.
6. Pendleton, W. R.: Methods for Preventing the Agglutination of Blood by Glucose Solutions, J. Lab. & Clin. Med. 12: 369, 1927.

THE OCCURRENCE OF A DOUBLE ZONE PHENOMENON IN ANTIHUMAN TISSUE SERUM*

Anderson Nettleship, M.D., Amsterdam, N. Y.

INTRODUCTION

DURING a group of experiments in which unperfused normal human kidney was injected into ducks, the duck serum was found to contain antibodies against human blood serum. This was not an unexpected result since the original material (antigen) contained some whole serum. Preliminary precipitin tests, however, showed such a well-defined double zone it was considered important to clarify its occurrence and if possible relate it to the proteins known to exist in human serum.

MATERIALS AND EXPERIMENTS

Normal human kidneys from acutely fatal trauma cases, in persons under the age of 40, showing no gross or microscopic changes beyond a mild degree of cloudy swelling, were removed within four hours of death; capsules stripped, and the kidney cut in long thin sections and frozen for twenty-four hours. After the pelvic fat was removed and the kidney ground fine, the pulp was suspended in minimal normal saline. Following an eight- to twelve-hour fat extraction (ether) in the cold, the water solution was precipitated with an excess of 80 per cent cold acetone. This precipitate, when resuspended in normal saline, proved fairly soluble. This precipitate was used to inject intravenously (3 c.c. to 4 injections) and intramuscularly (4 c.c. increasing to 10 c.c., 5 injections) in continuity every third day into adult Canton ducks. The fowls were bled on the tenth day following the last injection; the sera from 4 ducks were pooled and titrated against the following: (1) whole human blood precipitate (prepared similarly to the kidney precipitate except it had no fat extraction), (2) normal human sera, (3) whole *serum* precipitate (acetone precipitate in saline suspension), (4) erythrocyte cell stroma material, (5) hemoglobin, (6) globulin-free (Na_2SO_4 precipitated) serum. This wide variety of blood constituents was utilized in hopes of finding out what blood constituent was brought down in each precipitin zone. Results are given in Tables I, II, and III. Explanation of tables:

The antigen dilutions were made by mixing the antigen with normal saline, and diluting. The tests were run in 3 mm. diameter tubes, first putting the duck serum in and then the antigen with a capillary pipette. The tubes were water bathed four hours at 37.5° Centigrade and iceboxed over night. Readings were made in a reflected light against a concave mirror. In spite of attempting to layer the solutions, they did mix to some extent. Results are read from 0 to ++++ and where there was a ring it is shown thus ++°.

*Read by title, before the American Association of Pathologists and Bacteriologists, 42nd Annual Meeting, St. Louis, Missouri, April, 2, 1942.

This work was done while the author was Director of the Montgomery County Laboratories. At present, Passed Assistant Surgeon (R), U.S.P.H.S., Bethesda, Md.

Received for publication, February 18, 1943.

TABLE I

WHOLE HUMAN BLOOD PRECIPITATE (ANTIGEN) TITRATED AGAINST ANTIHUMAN DUCK SERUM

DIL. OF ANTIGEN	1.	2.	3.	4.	5.	6.
.030	0					
.021	0					
.020	0	+++		+	0	+
.019	0					
.018	0	+++		+	0	++
.016	0					
.015	0	++++	++++	++	++	+++
.014	0					
.013	0	++++	++++	+++	+++	+++
.011	0					
.010	+	+	+++	++	+	+++
.009	+++	++++	++++	++	++++	+++
.007	+	++	++++			+
.005	0	++				0
.003	0	+		+	+++	0
.002	0					0
.0019	0	0				++°
.0017	+					++
.0015	+					+++
.0013	++++					+++
.0011	++++		++++			+°
.0009	+++	+	++++	+	+++	+°
.0007	++	0		+	++	0
.0005	0			0		0
.0003	0			0		
.0001	0					

TABLE II

NORMAL HUMAN SERUM AND SERUM PRECIPITATE SUSPENSION TITRATED AGAINST ANTIHUMAN DUCK SERUM

DIL. OF ANTIGEN*	1.	2.	3.	DIL. OF ANTIGEN†	1.	2.	3.	4.
.030								
.021								
.020	0	++	+		0	0	0	+++
.019								
.018	0	++	++		0	0	0	+++
.016								
.015	0	+++	++°		++	0	0	+++
.014								
.013	0	++	++°		++++	++++	++++	+++
.011								
.010	+	0	++°		0	++++	++++	++++
.009	++	0	+++°		0	++++	++++	++++
.007	++	++	0		0	+++	+++	+++
.005	++	0	0		++++	+++	+++	++
.003	++	0	0		++++	++	++	++
.002	0	0	0		+++	+	+	0
.0019	0	+°	0		++	0	0	0
.0017	0	+°	+++		++	0	0	0
.0015	++	+°	++°		++	0	0	+
.0013	++	+°	++°		++	+++	+++	+
.0011	0	+°	++°		0	+++	+++	0
.0009	0	+°	+°		0	0	0	0
.0007	0	+°	+°		0	0	0	0
.0005	0	+°	+°		0	0	0	0
.0003								
.0001								

*Normal human serum.
†Whole serum precipitate.

TABLE III

ERYTHROCYTE STROMA, HEMOGLOBIN, AND GLOBULIN-FREE SERUM TITRATED AGAINST ANTIHUMAN DUCK SERUM

DIL. OF ANTIGEN*	1.	2.	3.	DIL. OF ANTIGEN†	1.	2.	3.	4.	DIL. OF ANTIGEN‡	1.	2.	3.	4.
.030	0												
.021													
.020	0	+++	0		+	++^{c}	0	+		+^{c}	0	0	0
.019	0												
.018	0	+++	+		+	++^{c}	0	++		+^{c}	0	0	0
.016	0		+										
.015	0	+++	+		+	++^{c}	+^{c}	+^{c}		0	0	0	0
.014	+		+										
.013	+	+++	+		+	+^{c}	+^{c}	+^{c}		0	0	+^{c}	0
.011	+												
.010	0	+++	+		++	+^{c}	+	++		+^{c}	0	+^{c}	0
.009	0	+++	+		+++	+^{c}	+	+		+^{c}	0	+^{c}	0
.007	0	++	+		++	0	+^{c}	+		+^{c}	0	0	+^{c}
.005	0	0	0		0	0	+^{c}	+		+^{c}	0	+^{c}	+^{c}
.003	0	0	0		0	0	+	0		+^{c}	+^{c}	+^{c}	0
.002	0	0	0		0	0	0	+		0	0	+^{c}	0
.0019	0	0	0		0	0	0	0		0	+^{c}	+^{c}	0
.0017	0	0	0		0	0	0	0		+^{c}	0	0	+^{c}
.0015	0	0	0		0	+	0	0		+^{c}	+^{c}	0	++^{c}
.0013	0	0	0		0	0	0	0		+^{c}	+	0	+++^{c}
.0011	0	0	+		0	0	0	+		+^{c}	+	+^{c}	++^{c}
.0009	0	0	0		+	0	0^c	0		+^{c}	+^{c}	0	0
.0007	±	0	0		±	0	0^c	0		0	+	0	+
.0005	0	0	0		0	0	0	0		0	0	0	+
.0003	0		0										
.0001													

*Erythrocyte stroma.
†Hemoglobin.
‡Serum albumin.

DISCUSSION

The duck serum tested contained a group of major antibodies against kidney substance, which are not discussed here, and minor antibodies. These minor antibodies, in varying dilutions, seemed to contain two entities, each with its distinctive optimal zone of precipitation. *When the duck antihuman serum was matched against whole blood precipitate, serum precipitate, or normal serum, the double zone made its appearance.* Red cell stromas (possibly globulins) gave a single precipitin zone as did hemoglobin. Their precipitins occurred in the zone of lower dilutions. Contrariwise, a globulin-free serum gave positive precipitins in higher dilutions. The precipitin reactions as seen from the tables were not always too clear cut, indicating that the precipitin method is more sensitive than the physical and chemical methods used to divide the compounds. As nearly as can be ascertained from published chemical data, the serum globulins are the larger, heavier molecular weight compounds, whereas, serum albumins are the smaller weight molecules.* These data, when fitted into the present precipitin system, show the larger molecular compounds combining in the lower dilutions, the smaller molecular weight compounds combining in the higher dilutions. Even though the antibodies are in the same solution, optimal zones of precipitation make their separation possible. The method may be useful in separating out unwanted antibodies where specific tissue-antibodies are to be studied.

*Svedberg, T., and Eriksson-Quensel, I. B.: Tabulae Biological Periodicae, V (4), 352 (1935-36). Quoted from Schmidt, C. L. A., The Chemistry of the Amino Acids and Proteins, Springfield, Ill., 1938, Charles C Thomas.

SUMMARY

Because of optimal precipitin conditions, it was possible to discover, in the minor antibody group of duck antihuman kidney serum, a double zone phenomenon. This phenomenon appears to rest upon the differential chemical structures involved. The higher molecular weight substances, the globulins, combine in the zone of lower dilutions. The smaller molecular weight albumins combine in the higher dilutions.

HEMATOLOGY OF THE PERIPHERAL BLOOD AND BONE MARROW OF THE DOG*

EDWARD J. VAN LOON, PH.D., AND BYRON B. CLARK, PH.D.
ALBANY, N. Y.

WITH THE TECHNICAL ASSISTANCE OF DOROTHY BLAIR

NORMAL values for peripheral blood of dogs have been reported by a number of investigators[1-13] (and others); however, the available data is still comparatively small. Only three reports of differential bone marrow counts were found.[14-16] In the course of several investigations considerable data have been obtained on normal values for peripheral blood of the dog together with some observations on bone marrow, and we are presenting a summary of this data.

METHODS

Adult mongrel dogs of both sexes weighing 8 to 25 kg. were used and all received our standard kennel ration. The animals were trained to be tied down and were allowed to remain quiet for some time before withdrawing the blood samples, which were obtained from the femoral artery at approximately the same hour of the morning. A mixture of dried ammonium and potassium oxalate was used as the anticoagulant. The blood was drawn into Thoma certified pipettes and diluted with Hayem's solution for the erythrocyte counts, and 1 per cent acetic acid solution tinted with gentian violet for the leucocyte counts. After hand shaking the pipettes for at least three minutes, a few drops were discarded, the hemocytometers filled (certified Levy-Hausser chambers with Neubauer rulings), and duplicate counts were made.

Smears for the leucocyte differential and reticulocyte counts were made initially by both the slide and coverslip methods, but the latter is now used exclusively since a better distribution of cells is obtained. Two hundred cells were counted for the leucocyte differential. The smears for reticulocytes were prepared after staining with brilliant cresyl blue, and at least 500 red cells were counted. Occasionally the wet mount technique was employed. Wright's stain was used for the leucocyte smears and for counterstaining the reticulocyte

*From the Department of Physiology and Pharmacology, Albany Medical College, Union University.
Received for publication, March 1, 1943.

smears. Cell volume was determined in duplicate with Wintrobe tubes.[17] Hemoglobin was determined by the Evelyn spectrophotometric method.[18]

Bone marrow samples were obtained immediately following sacrifice of the animals. Three different types of preparations were made: (1) Transverse sections were sawed from the upper third of the femur, and the marrow was shelled out of the bony ring, fixed in Zenker's formalin, sectioned, and stained with hematoxylin and eosin. (2) Impression imprints were made from femur marrow tissue and stained with Wright's or Giemsa's stain. (3) Marrow samples extruded from rib sections were diluted with heparin or heparinized serum, cover slips smears prepared, rapidly dried, and stained with Wright's or Giemsa's stain.[19] Differential counts of 500 or 1000 cells were made on the two latter types of preparations.

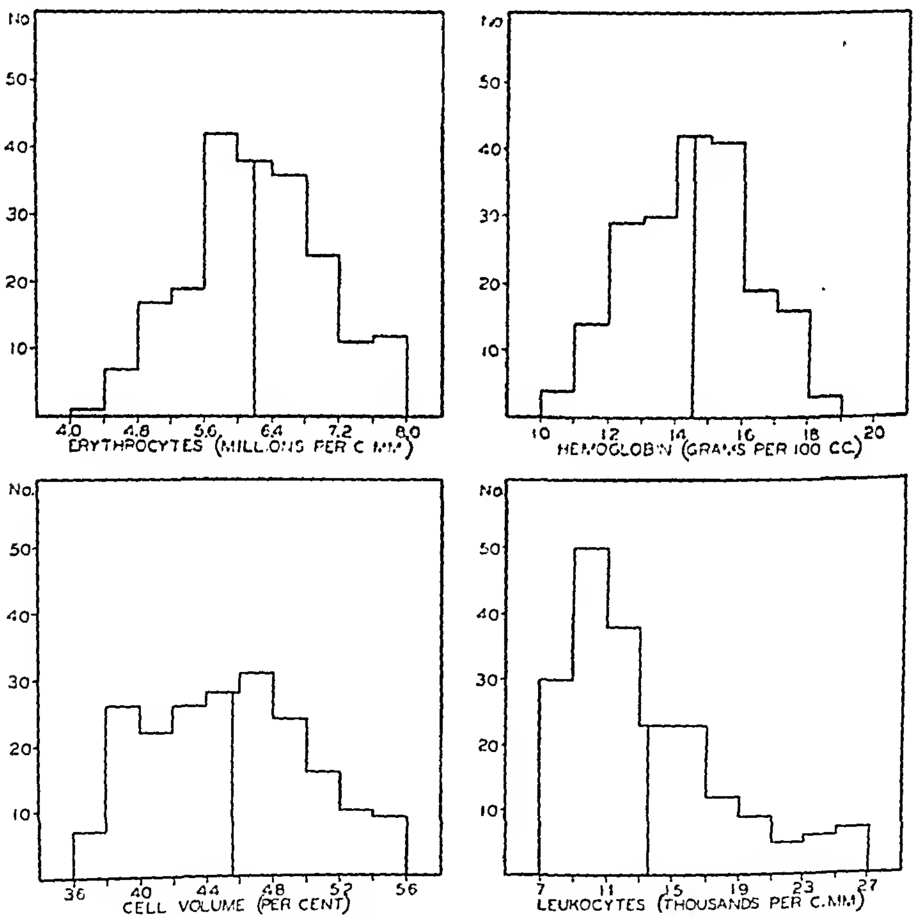


Fig. 1.—Frequency histograms of the hematology of 81 dogs.

RESULTS

Our observations on peripheral blood were made on 81 dogs and include 207 erythrocyte counts, 120 reticulocyte counts, 203 leucocyte counts, 199 determinations of cell volume, 198 determinations of hemoglobin, and 196 differential leucocyte counts. The observations on most individual animals were made during a period of one to two months; in 11, however, they extended over

a period of several months. The observations on the red and white blood cell counts, hemoglobin, and cell volume have been grouped in 2 ways, statistically analyzed, and are presented in Table I. All determinations on the 81 dogs are included in part A, and frequency histograms are shown in Fig. 1. Calculation from the mean values reveals a mean corpuscular volume of 73.4 cubic microns, a mean corpuscular hemoglobin of 23.7 micromicrograms, and a mean corpuscular hemoglobin concentration of 32.3 per cent. In part B the results of taking a single observation on each dog are shown; the last determination which had been made was arbitrarily chosen. These data are not significantly different, thus showing that an unequal number of determinations on some of the animals did not materially influence the analysis in part A. We noted that there was a general tendency for the erythrocyte, hemoglobin, and cell volume values to decrease slightly when the observations were extended over a long period. This trend was more definite in the first few weeks and was considered a result of training.

TABLE I

STATISTICAL ANALYSIS OF PERIPHERAL BLOOD HEMATOLOGY

		MEAN	STANDARD DEVIATION	PROBABLE ERROR OF MEAN	COEFFICIENT OF VARIATION	NO. OF OBSERVATIONS
A	Erythrocytes $\times 10^6$ per c.mm.	6.202	0.98	0.046	15.8	207
	Hemoglobin Gm. per 100 c.c.	14.56	2.05	0.098	14.1	198
	Cell Volume Per Cent	45.60	5.79	0.277	12.7	199
	Leucocytes $\times 10^3$ per c.mm.	13.523	4.48	0.212	33.1	203
B	Erythrocytes $\times 10^6$ per c.mm.	6.298	0.74	0.055	11.6	81
	Hemoglobin Gm. per 100 c.c.	14.65	2.27	0.170	15.5	81
	Cell Volume Per Cent	45.56	5.10	0.403	11.2	81
	Leucocytes $\times 10^3$ per c.mm.	13.110	4.43	0.336	33.8	81

TABLE II

COMPARISON OF MEAN VALUES REPORTED FOR HEMATOLOGY OF DOG BLOOD

NO. OF DOGS	HB	R.B.C.	CELL VOLUME	RETICULOCYTES	W.B.C.	DIFFERENTIAL					REF. NO.
						P	L	M	E	B	
	GM. %	PER CENT MM. $\times 10^6$	%	%	PER CENT MM. $\times 10^3$						
25	15.8	7.00	45.2								1
32	14.1	7.17	47.7		8.76						2
43-50	16.0	6.87	46.9								3
54	14.6	7.02	47.3								4
34	13.6	6.45	44.3	0.4	14.18						5
5-12	13.5	6.48		1.1	14.40						6
8-15		7.22			19.30	64.6	22.2	4.4	8.9		7
20		6.21			9.53	65.7	21.0	6.8	5.3		8
11-47	15.5	5.97		0-1.4	15.92	66.7	22.1	6.8	5.1		9
12		6.21			17.05	77.4	13.2	6.1	3.3		10
60	13.0	6.16	38.6	1.8	11.16	74.0	20.0	4.0	2.0		11
566	13.9	7.22			11.84	69.0	20.0	6.1	5.0	0.7	12
31	15.1	6.20			11.46	71.8	21.7	0.7	5.4		13
81	14.6	6.20	45.6	0-1.5	13.52	71.8	20.1	3.7	4.4	rare	*

*Mean values found by this laboratory.

The mean values of our data, compared with some of those reported in the literature, are shown in Table II. Our results are in general agreement with those of Bruner, et al.,[5] Mayerson,[11] Morris, et al.,[12] and others.

It was noted in making the reticulocyte count by both the wet and dry methods that there were many instances in which no reticulocytes were seen in counting 500 erythrocytes. Further examination of the smears, however, always revealed the presence of some reticulocytes.

We have observed, as others have reported, that no significant difference exists between the values obtained for males and females, for the different breeds, and that age apparently has little or no effect after the animals are 10 to 12 months old.

The mean and range of bone marrow differential counts on rib smears and femur imprints on the same eight dogs are shown in Table III. The impression imprints probably give a more nearly physiologic distribution of marrow cells. These preparations are difficult to prepare, however, do not stain well, and the counts obtained are somewhat selected, since usually only those cells on the periphery of each imprint can be differentiated. Smears from extruded rib marrow are easy to prepare, stain well, and cells over the entire smear are readily differentiated. It is our impression that representative marrow counts are obtained from these smears.

TABLE III

BONE MARROW DIFFERENTIAL COUNTS

TYPE OF CELL	RIB SMEARS		FEMUR IMPRINTS	
	MEAN	RANGE	MEAN	RANGE
Myeloblasts	0.6	0.2- 1.0	0.5	0.2- 1.0
Promyelocytes	1.6	0.7- 2.8	0.8	0.2- 1.1
Myelocytes	6.0	2.7-10.0	4.6	1.7- 8.0
Metamyelocytes	3.4	1.1- 4.6	3.3	2.0- 4.3
Bands, Neutrophilic	11.7	6.8-17.4	12.6	10.3-18.7
Segmenters, Neutrophilic	30.1	16.8-44.2	34.7	30.1-39.7
Eosinophils	2.0	0.1- 3.8	2.0	0.2- 3.3
Monocytes	0.2	0- 0.3		0- 0.1
Lymphocytes	0.9	0.2- 2.7	0.7	0- 3.4
Pronormoblasts	0.6	0.2- 1.2	0.3	0- 0.7
Normoblasts, Basophilic	7.8	6.4- 9.9	6.1	4.2- 8.4
Normoblasts, Polychromatic	16.4	11.0-26.0	16.5	13.3-19.8
Normoblasts, Orthochromatic	17.4	8.9-25.8	16.1	7.6-21.1
Megakaryocytes	0.5	0- 1.4	0.2	0- 0.3
Unclassified		0.8- 1.4		0.4- 3.1

Comparison of the data on rib smears with those on the femur imprints reveals a close similarity. Stasney and Higgins[15] have already pointed out that marrow from the rib, proximal femur, and middle femur show essentially the same general trend in the relative percentage of distribution of cells, although the myeloid-erythroid ratio is somewhat higher in the rib. Our mean myeloid-erythroid ratio is slightly over one (range 0.6 to 2.4), thus agreeing with the data of Mulligan[16] and Alexandrov.[14] Stasney and Higgins found the myeloid-erythroid ratio consistently below one; however, their mean erythrocyte count was only slightly above 5.0 million. These observations lend further validity to the rib smear technique.

Histologic examination of the femur marrow sections revealed a wide variation in total cellularity. In general, active hematopoietic tissue was found around the periphery of the section and as small islands among the fat cells which filled the center of the section.

SUMMARY

A summary and statistical analysis of the data on the peripheral blood hematology of 81 dogs are presented. Differential cell counts of rib and femur marrow preparations from 8 dogs are included.

The authors are grateful to Dr. J. L. Schwind of the Department of Anatomy for many helpful suggestions.

REFERENCES

1. Powers, J. H., Bowie, M. A., and Howard, I. M.: Some Observations on Blood of Normal Dog With Special Reference to Total Volume, Am. J. Physiol. 92: 665, 1930.
2. Leichsenring, J. M., Biester, A., Hönig, H. H., Furnas, S. M., Foss, E. S., and Routt, M. V.: Blood Regeneration Studies. II., Am. J. Physiol. 99: 391, 1932.
3. Ashley, A., and Guest, G. M.: Distribution of Blood Phosphorus After Suppression of Renal Function, J. Clin. Investigation 13: 219, 1934.
4. Wintrobe, M. M., Shumacker, Jr., H. B., and Schmidt, W. J.: Values for Number, Size and Hemoglobin Content of Erythrocytes in Normal Dogs, Rabbits, and Cats, Am. J. Physiol. 114: 502, 1936.
5. Bruner, H. D., and Wakerlin, G. E.: Blood Picture of Normal Dog, Proc. Soc. Exper. Biol. & Med. 36: 667, 1937.
6. Castrodale, D., Bierbaum, O., Helwig, E. B., and MacBryde, C. M.: Comparative Studies of the Effects of Estradiol and Stilbestrol on the Blood, Liver, and Bone Marrow, Endocrinology 29: 363, 1941.
7. Dawson, P. M.: Effects of Venous Hemorrhage and Intravenous Infusion in Dogs, Am. J. Physiol. 4: 1, 1900.
8. Busch, F. C., and Van Bergen, C.: Differential Counts of Leucocytes in Dog Blood, J. Med. Research 8: 408, 1902.
9. Musser, J. H., and Krumbhaar, E. B.: Studies of Blood of Normal Dog, Folia haemat. 18: 576, 1914.
10. Kohanawa, C.: Beiträge zur vergleichenden Morphologie des Blutes der gesunden Haussäugetiere, Folia haemat. 36: 174, 1928.
11. Mayerson, H. S.: Blood Cytology of Dogs, Anat. Rec. 47: 239, 1930.
12. Scarborough, R. A.: Blood Picture of Normal Laboratory Animals, Yale J. Biol. & Med. 3: 359, 1931.
13. Morris, M. L., Allison, J. B., and Green, D. F.: Blood Cytology of the Normal Dog, J. Lab. & Clin. Med. 25: 353, 1940.
14. Alexandrov, A. J.: Die Morphologie des Sternumpunktates von Hunden, Folia haemat. 41: 428, 1930.
15. Stasney, J., and Higgins, G. M.: A Quantitative Cytologic Study of the Bone Marrow of the Adult Dog, Am. J. M. Sc. 193: 462, 1937.
16. Mulligan, R. M.: Quantitative Studies on the Bone Marrow of the Dog, Anat. Rec. 79: 101, 1941.
17. Wintrobe, M. M.: Macroscopic Examination of the Blood: Discussion of Its Value and Description of the Use of a Single Instrument for the Determination of Sedimentation Rate, Volume of Packed Cells, Leukocytes and Platelets, and of Icterus Index, Am. J. M. Sc. 185: 58, 1933.
18. Evelyn, K. A., and Malloy, H. T.: Microdetermination of Oxyhemoglobin, Methemoglobin, and Sulfhemoglobin in a Single Sample of Blood, J. Biol. Chem. 126: 655, 1938.
19. Isaacs, R.: Physiologic Histology of Bone Marrow; Mechanism of Development of Blood Cells and Their Liberation Into Peripheral Circulation, Folia haemat. 40: 395, 1930.

EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE ON MICE INFECTED WITH HEMOLYTIC STREPTOCOCCUS, PNEUMOCOCCUS, AND STAPHYLOCOCCUS AUREUS*

George W. Raiziss, Ph.D., M. Severac, M.D., and J. C. Moetsch, Philadelphia, Pa.

SUBSTITUTION of sulfanilamide in the sulfonamide group by replacement of one of the hydrogens by various heterocyclic compounds resulted in some cases in a substantial enhancement of therapeutic activity. The heterocyclic part of sulfadiazine, therapeutically a very active compound, consists of the pyrimidine ring where the 2 nuclear nitrogens are in the meta position to each other. Raiziss and Clemence[1] described a derivative containing a heterocyclic with 2 nuclear nitrogens in the para position. The same product was prepared independently by Elligson.[2] This derivative was obtained by the interaction of para-acetylaminobenzene sulfonyl chloride with 2-aminopyrazine. For the sake of brevity we named this product sulfapyrazine.

Sulfapyrazine has already been investigated clinically in the treatment of pneumonia and reported by Ruegsegger, Hamburger, Turk, Spies, and Blankenhorn.[3] The authors stated that 22 selected patients who received the drug by mouth showed prompt improvement and ultimate recovery with no significant signs of toxicity.

We are presenting studies on toxicity and blood levels and also on the therapeutic effect of sulfapyrazine in mice infected with various pathogenic organisms.

TOXICITY

Mice were fed a drug food diet containing ¼, ½, ¾, and 1 per cent of sulfapyrazine or sulfadiazine for a fourteen-day period. We have determined that mice consume on an average 4 Gm. per day of the drug diet. This would be equivalent to 0.010 Gm. of drug in ¼ per cent diet; 0.020 Gm. in ½ per cent; 0.030 Gm. in ¾ per cent; and 0.040 Gm. in 1 per cent. Table I shows that ¼ and ½ per cent drug diets containing either sulfapyrazine or sulfadiazine were well tolerated when fed for a period of fourteen days. Three-fourths per cent diet was tolerated only by 60 per cent of animals; 1 per cent diet of sulfapyrazine was tolerated by 50 per cent of animals at the end of fourteen-day period, while on a 1 per cent sulfadiazine drug diet all animals died after nine days of feeding.

BLOOD LEVEL DETERMINATIONS

Blood levels were determined by Bratton and Marshall's method.[4] From Table II it is apparent that sulfapyrazine on various drug diets produces com-

*From the Dermatological Research Laboratories, Philadelphia, Division of Abbott Laboratories, North Chicago.

A preliminary paper was presented before the Federation of American Societies for Experimental Biology at Boston, Mass., March 27, 1942, by the senior author (G. W. R.)

Received for publication, March 1, 1943.

TABLE I

Chronic Toxicity Feeding Experiments Drug Diet Administered for 14 Days

COMPOUND	PER CENT DRUG WITH FOOD*	NO. MICE USED	PERCENTAGE OF SURVIVALS IN DAYS													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sulfapyrazine	¼†	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	½	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	¾	10	100	100	100	90	90	90	90	90	90	70	60	60	60	60
	1	10	100	100	90	80	60	50	50	50	50	50	50	50	50	50
Sulfadiazine	¼	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	½	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	¾	10	100	100	100	100	100	90	90	90	90	70	70	70	60	60
	1	10	100	100	100	90	70	30	20	10	10	0				

*Mice consume on an average 4 Gm. per day of the drug diet.

†¼ per cent diet contains 0.010 Gm. of drug per day: ½ per cent, 0.020 Gm.; ¾ per cent, 0.030 Gm.; and 1 per cent, 0.040 Gm.

paratively high blood levels in mice ranging from 7.2 to 32 mg. per 100 c.c., which, however, are lower than those obtained with the respective sulfadiazine feedings.

COMPARATIVE THERAPEUTIC EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE

In all of the therapeutic experiments drugs were administered to mice in food according to the method described by Litchfield, White, and Marshall.[5]

Mice were infected with the *Streptococcus hemolyticus* strain C203 using 1000 minimum lethal doses containing from 5 to 10,000 bacteria. According to Table III sulfapyrazine exerted a better therapeutic effect than sulfadiazine on mice with streptococcus infection fed the ¼ per cent drug diet. On the ½ per cent drug diet sulfadiazine gave better results than sulfapyrazine. Under the same condition, sulfanilamide proved to be less effective.

TABLE II

AVERAGE OF DAILY RECORDED BLOOD LEVELS IN MICE FED DRUG DIET FOR 10 DAYS

PER CENT DRUG IN FOOD	BLOOD CONCENTRATIONS SULFAPYRAZINE MG. PER 100 C.C.*	FREE DRUG SULFADIAZINE MG. PER 100 C.C.*
¼	7.2	15
½	10.4	26
¾	28	38
1	32	43

*The averages represent tests using from 4 to 6 mice.

TABLE III

COMPARATIVE THERAPEUTIC EFFECT OF SULFAPYRAZINE AND SULFADIAZINE DRUG ADMINISTERED WITH FOOD FOR TEN DAYS

STREPTOCOCCUS HEMOLYTICUS INFECTION, STRAIN C203

PER CENT DRUG WITH FOOD	DRUG	NO. OF MICE USED	PERCENTAGE OF SURVIVALS DAYS 1	2	3	4	5	6	7	14	21	28
¼	Sulfapyrazine	25	100	100	100	100	100	100	100	96	96	96
	Sulfadiazine	25	96	92	92	92	92	92	92	88	84	84
½	Sulfapyrazine	80	91	91	82	80	76	75	70	59	59	50
	Sulfadiazine	80	100	98	84	84	81	78	78	74	71	71
	Sulfanilamide	25	84	84	68	60	60	60	60	56	56	56
	Controls	27	4	0								

Heart cultures of autopsied mice who died before the 28th day invariably were positive for Streptococci.

In the experiments on Type I pneumococcus infection we used strain M-36.[6] Mice were infected with 10 minimum lethal doses, the total content of inoculum being about 100 bacteria. This strain proved to be of high virulence. As seen from Table IV, 88 per cent of mice on the ¼ per cent sulfapyrazine diet survived seven days. After fourteen days the survival rate dropped to fifty per cent. On ¼ per cent sulfadiazine diet, 33 per cent of mice survived seven days and after fourteen days 23 per cent, indicating a better effect for sulfapyrazine. On a ½ per cent drug diet sulfadiazine was somewhat less effective than sulfapyrazine.

In our experiments on Type II pneumococcus infection, we used strain M-48 as described in previous publication.[6] Mice were infected intraperitoneally with 10 minimum lethal doses containing about 250 bacteria.

From Table IV one can see that mice fed ¼ per cent of sulfapyrazine diet showed 96 per cent survivals after four days, 44 per cent after fourteen days and 40 per cent after twenty-eight days of observation.

Under the same conditions, sulfadiazine therapy resulted in 33 per cent survivals after four days and only 5 per cent survivals after fourteen and twenty-eight days. On ½ per cent diets both drugs after twenty-eight days gave equally good therapeutic results.

Pneumococcus Type III proved to be more resistant to drugs than Type II. We used 10 minimum lethal doses which contained about 200 bacteria of strain M-50. With a ¼ per cent diet, 20 per cent of the mice fed sulfapyrazine survived three days, while at the end of twenty-eight days 5 per cent survived. Mice given ¼ per cent sulfadiazine diet showed about the same results with a survival rate of 20 per cent after three days and 7 per cent at the end of twenty-eight day period.

TABLE IV

THERAPEUTIC EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE IN MICE
DRUG ADMINISTERED WITH FOOD FOR TEN DAYS

PNEUMOCOCCUS	PER CENT DRUG WITH FOOD	COMPOUND	NO. MICE USED	PERCENTAGE OF SURVIVALS DAYS									
				1	2	3	4	5	6	7	14	21	28
Type I Strain M-36	¼	Sulfapyrazine	40	98	98	95	88	88	88	88	50	50	50
	½	Sulfapyrazine	40	98	85	85	85	85	83	83	73	70	70
	¼	Sulfadiazine	40	100	55	38	35	33	33	33	23	23	23
	½	Sulfadiazine	40	98	95	95	95	93	93	93	63	55	55
		Controls	18	2	0								
Type II Strain M-48	¼	Sulfapyrazine	50	96	96	96	96	90	90	86	44	40	40
	½	Sulfapyrazine	40	100	100	93	93	90	88	85	60	55	55
	¼	Sulfadiazine	40	95	75	50	33	25	25	15	5	5	5
	½	Sulfadiazine	40	100	100	100	100	93	93	88	58	55	55
		Controls	23	4	0								
Type III Strain M-50	¼	Sulfapyrazine	20	95	35	20	10	10	10	10	10	5	5
	½	Sulfapyrazine	40	100	90	70	65	58	58	55	40	38	38
	¼	Sulfadiazine	15	87	33	20	13	7	7	7	7	7	7
	½	Sulfadiazine	40	100	85	55	50	43	33	30	28	25	25
		Controls	17	18	0								

Heart cultures of autopsied animals invariably were positive for Pneumococci.

On the ½ per cent diet at the end of three days sulfapyrazine had 70 per cent, while sulfadiazine had only 55 per cent survivals. At the end of twenty-eight days, 38 per cent survivals were observed with sulfapyrazine and only 25 per cent with sulfadiazine. In this case sulfapyrazine showed better therapeutic effect.

Staphylococcus aureus infection was produced in mice by the intravenous administration (saphenous vein) of 0.2 c.c. of a saline suspension containing about 8 billion bacteria. This culture had five passages through mice prior to its use. As seen from Table V, 50 per cent of control mice died at the end of seventy-two hours and the rest died seven days after infection.

On a ¼ per cent sulfapyrazine diet, 40 per cent of the animals survived for eleven days and 30 per cent survived fourteen days, showing a slightly better effect than sulfadiazine.

On a ½ per cent drug diet, sulfadiazine proved to be better.

TABLE V

COMPARATIVE THERAPEUTIC EFFECTS OF SULFAPYRAZINE AND SULFADIAZINE
DRUG ADMINISTERED *WITH* FOOD FOR TEN DAYS

STAPHYLOCOCCUS AUREUS INFECTION, STRAIN 209

PER CENT DRUG WITH FOOD	DRUG	NO. MICE USED	PERCENTAGE OF SURVIVALS DAYS													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1/4	Sulfapyrazine	10	100	100	100	90	80	80	80	80	70	60	40	40	30	30
	Sulfadiazine	10	100	100	90	90	80	80	60	50	50	40	30	20	20	20
1/2	Sulfapyrazine	10	100	100	90	70	60	60	30	30	30	20	0			
	Sulfadiazine	10	100	100	90	90	80	80	80	80	80	70	70	60	50	40
	Controls	10	100	70	50	30	20	10	0							

Cultures of kidneys of autopsied animals were positive for *Staphylococcus aureus.*

DISCUSSION

(a) Sulfapyrazine, an isomer of sulfadiazine, by this and further investigations may prove to possess certain advantages over sulfadiazine in clinical application and, therefore, may be valuable.

(b) We have established that blood levels of mice kept on drug diets containing sulfapyrazine are generally lower than those of sulfadiazine, being higher than blood levels obtained with other sulfanilamide derivatives.

(c) This report is the first, according to our knowledge, of an extensive study of sulfapyrazine by the food drug diet method on various types of pneumococcus.

(d) By a careful series of experiments, we established that sulfapyrazine has either equal or somewhat better therapeutic effect on food drug diets at lower blood levels than sulfadiazine.

CONCLUSIONS

1. Mice tolerate sulfapyrazine and sulfadiazine on a 1/4 and 1/2 per cent food diet equally well, while a 3/4 and 1 per cent drug diet sulfapyrazine is somewhat better tolerated than sulfadiazine.

2. Blood levels on 1/4 and 1 per cent drug diets ranged from 7.2 to 32 mg. per 100 c.c.

3. In beta-hemolytic streptococcic infection both drugs were of equal therapeutic effectiveness.

4. In Type I Pneumococcus infection, mice fed on 1/4 and 1/2 per cent sulfapyrazine drug diets showed a better survival rate.

5. In Type II pneumococcus infection, sulfapyrazine showed a greater survival rate, than sulfadiazine on 1/4 per cent and equal on 1/2 per cent drug diets.

6. In Type III pneumococcus infection both drugs were equally effective on the 1/4 per cent diet, while sulfapyrazine gave better results on a 1/2 per cent diet.

7. According to our experiments, in *Staphylococcus aureus* infection, sulfapyrazine and sulfadiazine proved to be equally effective.

REFERENCES

1. Raiziss, G. W., Clemence, L. W., and Freifelder, M.: N1-Heterocyclic Sulfanilamide Derivatives, J. Am. Chem. Soc. 63: 2739, 1941.

2. Elligson, R. C.: Sulfapyrazine, Sulfapyrimidine and Sulfadiazine, J. Am. Chem. Soc. 63: 2524, 1941.
3. Ruegsegger, J. M., Hamburger, M., Turk, A. S., Spies, T. D., and Blankenhorn, M. S.: The Use of 2-Sulfanilylaminopyrazine in Pneumococcal Pneumonia, Am. J. M. Sc. 202: 432, 1942.
4. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. 128: 537, 1939.
5. Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr.: Experimental Basis for Method for Quantitative Evaluation of Effectiveness of Chemotherapeutic Agents Against Streptococcus Infection in Mice, J. Pharmacol. & Exper. Therap. 67: 437, 1939.
6. Raiziss, G. W., Severac, M., Moetsch, J. C., and Clemence, L. W.: Protective Effect of Sulfapyridine, Sodium Sulfapyridine and Sulfanilamide in Pneumococcus Infection Types I, II and III, Proc. Soc. Exper. Biol. & Med. 42: 12, 1939.

A NEW METHOD FOR THE EVALUATION OF DRUGS AFFECTING THE REACTION OF MICE TO PAIN STIMULATION*

KENNETH KUETER, B.S., AND RICHARD K. RICHARDS, M.D., NORTH CHICAGO, ILL.

IN recent years a number of new methods for the testing of analgesic drugs in animals has been reported. Without claim for completeness we mention here the methods of Macht and Macht,[1] d'Amour and Smith,[2] and of Andrews and Workman,[3] the last two methods being modifications of the principle developed by Hardy, Wolff, and Goodell.[4] In our experience, results can be obtained with all of them. We found that there was little consistency, however, inasmuch as certain drugs were found relatively more potent with one of the methods than with another. On account of this we employ as a rule several procedures in estimating analgesic properties.

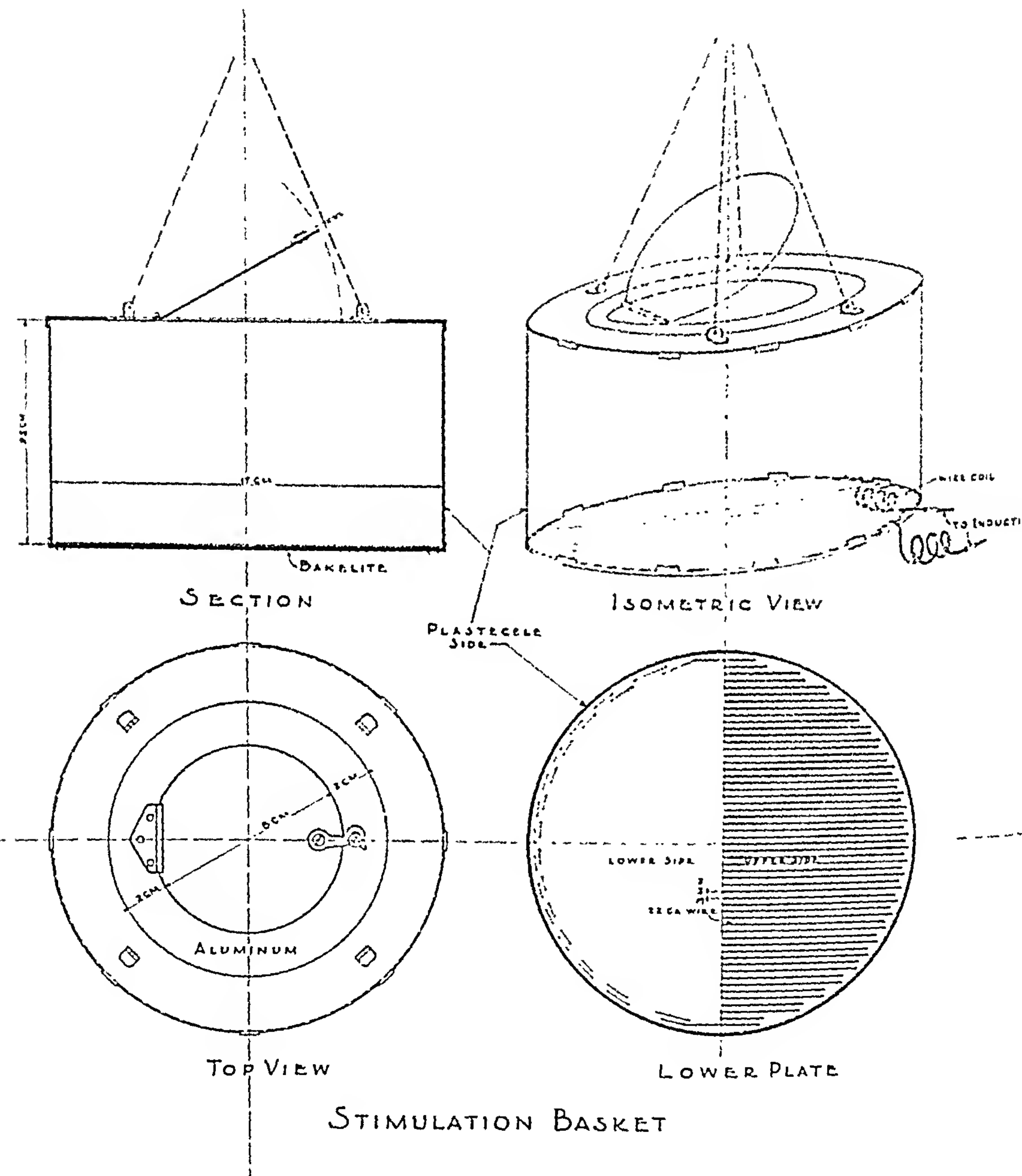
The necessity of immobilizing the animal during the test represents, in our opinion, a common disadvantage of these methods. Since it is not certain whether any method of determining analgesia in animals measures "pain" in the sense of the human quality or just a reflex, we believe the experimental conditions should enable the animal to be as unrestricted in its movements as possible.

This demand is satisfied in a procedure used by Straub and Triendl[5] and based upon a method developed by Forst,[6] which consists essentially of having a mouse move about freely on a disc and measuring the movements by means of a work adder; a clamp is then applied to the nose or rectum and the reaction is measured by the increased activity of the animal. We found it profitable to substitute the continuous stimulation caused by the clamp by an intermittent stimulation which can also be easily varied in degree. Our procedure does not require complicated or expensive apparatus and has proved valuable in our hands in combination with other methods.

METHOD

Mice are used for this test. The animals are stimulated through their feet in a basket made of plasteele and aluminum with a bottom made of lightweight

*From the Department of Pharmacological Research, Abbott Laboratories.
Received for publication, March 8, 1942.



17 CM
Bakelite
Section
Isometric View
Wire coil
To inducti
Plastecele Side
Aluminum
Lower side
Upper side
Top View
Lower Plate
Stimulation Basket

Fig. 1.

bakelite. The basket resembles a small hatbox. It is 17 cm. in diameter and 9.5 cm. in height. The bottom of the basket is covered with rows of fine copper wire about 3.0 mm. apart. These wires are strung across the bottom so that one wire is positive and the adjacent one negative. This is accomplished by stringing one continuous wire in rows about 6 mm. apart and then stringing another wire in between the rows of the first strung wires. Care must be taken so that the two wires are well insulated from each other. (Fig. 1 shows how this is accomplished.) The basket is suspended from a bellows made by stretching a condom over a large-sized heart chamber. The basket hangs by means of strings and a wire hook made fast in a small, square cardboard and fastened to the condom with rubber cement. The heart chamber is connected to a tambour and writing arm with rubber tubing. The tambour is held in place by a clamp and ring stand. Any movement in the basket sends air impulses through the tubing to the tambour and arm. Movements of the animal are recorded on a slowly moving kymograph. The basket is connected to the secondary coil of a Harvard inductorium, and the primary (4 volts) current is made intermittent by means of a Harvard electric time clock or metronome. The physical considerations of the Harvard inductorium are explained in detail by Macht and Macht.[1] (Fig. 2 shows the complete apparatus.)

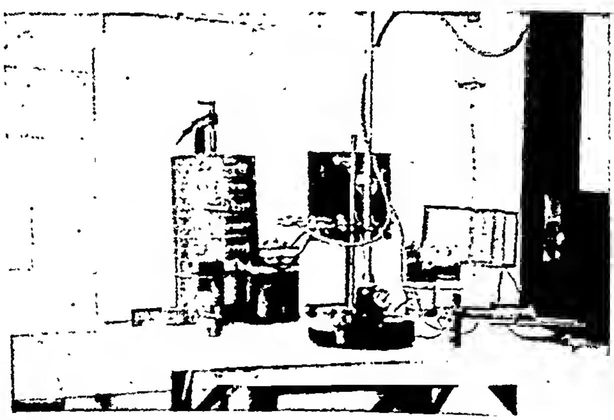
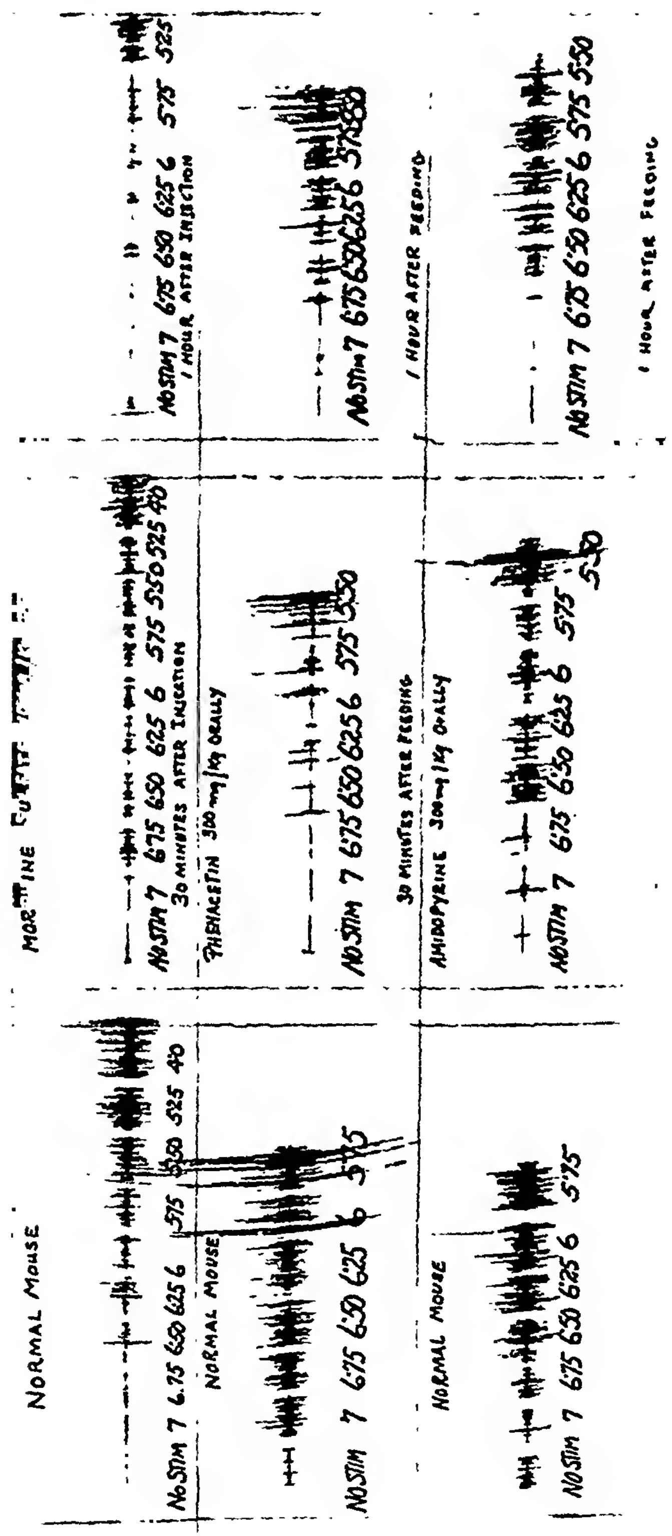


Fig. 2.

At least two mice are run on a dose level. The animals are first tested as normal mice. A tracing is always made with no stimulation, covering about one inch of paper. Then, the secondary coil is set at 7 cm., and the time clock is set in motion. A short tracing is made and the current is shut off. The secondary coil is then moved to 6.75 cm. A tracing is made, the current interrupted, the coil shifted to 6.5 cm., and so on, in 0.25 to 0.5 cm. intervals. As the stimulus becomes stronger, the mice start to make jumping movements which increase to a maximum, usually at about 5.5 to 4.0 cm. secondary coil distance. There is a considerable variation in the sensitivities of the individual animals that has to be taken into consideration for the evaluation of an analgesic. Mice which fail to show an increment of reaction with increased stimulation or are too restless are discarded.



NORMAL MOUSE
NO STIM 7 6.75 6.50 6.25 6 5.75 5.50 5.25 4.0
NORMAL MOUSE
NO STIM 7 6.75 6.50 6.25 6 5.75
NORMAL MOUSE
NO STIM 7 6.75 6.50 6.25 6 5.75
30 MINUTES AFTER INJECTION
PHENACETIN 300 mg/kg ORALLY
30 MINUTES AFTER FEEDING
AMIDOPYRINE 300 mg/kg ORALLY
1 HOUR AFTER INJECTION
1 HOUR AFTER FEEDING
1 HOUR AFTER FEEDING

Fig. 3.

TABLE I

EFFECT OF ORAL ADMINISTRATION OF ANALGESICS ON THE PAIN THRESHOLD

MOUSE NO.	DRUG ADMINISTERED	DOSE MG./KG.	PAIN THRESHOLD NORMAL CM.	15 TO 30 MIN. AFTER DOSE CM.	1 TO 2 HOURS AFTER DOSE CM.
1	Phenacetin	300	6.75	6.75	6.0
2	Phenacetin	750	6.5	6.5	6.0
3	Phenacetin	750	7.0	6.0	5.5
4	Phenacetin	1000	7.0	5.0	4.0
5	Amidopyrine	250	7.0	5.5	6.0
6	Amidopyrine	300	7.0	6.0	5.5
7	Aspirin	500	6.5	6.5	6.5
8	Nembutal	55	7.0	7.0	6.5
9	Nembutal	75	7.0	4.0	4.0
10	Nembutal*	110	7.0	4.0	0.0

*Animal was in surgical anesthesia.

TABLE II

EFFECT OF INTRAPERITONEAL INJECTION OF MORPHINE ON THE PAIN THRESHOLD

MOUSE NO.	DRUG ADMINISTERED	DOSE MG./KG.	PAIN THRESHOLD NORMAL CM.	15 TO 30 MIN. AFTER DOSE CM.	1 TO 2 HOURS AFTER DOSE CM.
1	Morphine SO_4	4	7	6.5	6.5
2	Morphine SO_4	6	7	6.0	6.0
3	Morphine SO_4	6	7	6.0	6.0
4	Morphine SO_4	10	7	5.5	6.0
5	Morphine SO_4	10	7	5.25	5.25
6	Morphine SO_4	10	7	6.5	5.0
7	Morphine SO_4	10	7	5.0	4.5
8	Morphine SO_4	10	6.5	5.5	4.5
9	Morphine SO_4	15	7	5.5	5.5
10	Morphine SO_4	20	7	5.5	5.5

After the normal reactions of the mouse have been recorded, the drug under investigation is administered either by mouth or by intraperitoneal injection. For the first test we give usually 25 to 50 per cent of the L.D.50. Later the dose has to be adjusted according to experience. Tests are made in intervals of 20, 30, or 60 minutes depending on the type of drug and method of administration. Fig. 3 shows tracings of such experiments, and a summary of the effects of a few well-known analgesics administered orally is given in Table I; the columns headed "Centimeter" show the reading of the secondary coil at which a definite stimulation was recorded after the drug. Necessarily, this criterion is somewhat subjective with the individual investigator, but if interpretations are always made on the same basis, the results become comparable. Table II shows the effects of morphine by intraperitoneal injections. Whereas the sensitivity of this method compares favorably with other procedures, we failed to obtain a definite analgesic effect with aspirin after oral administration with the chosen dose, but phenacetin and amidopyrine exhibited definite analgesic action. Nembutal was ineffective at 55 mg./kg., a dose not producing marked depression. Analgesia appeared with the definitely depressing dose of 75 mg./kg. and became complete with 110 mg./kg. In this stage no reaction was elicited. This proves that our method of stimulation produces central effects and not merely peripheral con-

traction by means of peripheral stimulation. Newer drugs which were tested and found analgesic by this procedure have proved to be effective in preliminary clinical investigations.

We are aware that, in testing of analgesic drugs, not only their central true analgesic action but also other effects have to be taken into consideration. Thus, morphine possesses a definite stimulating effect upon the spinal centers, an action which results in the well-known Straub phenomenon; however, in our test, signs of the spinal stimulation do not show up (*see Fig. 3, upper tracings*). The analgesic effect of aspirin, so conspicuous in humans, appears to be difficult to demonstrate in animals. This has been substantiated recently again by Smith, D'Amour, and D'Amour.[7] The barbiturates which exert a general cerebral depressing effect fail to influence selectively the pain centers, as is demonstrated by our failure to obtain analgesic effects with sedative doses; this is in agreement with clinical experience. Taking all these factors in consideration, one has to recognize the limitations of new methods endeavoring to measure a particular property in the presence of other factors which will influence a test.

We believe that the above described method will be of help to investigators in this field in conjunction with other procedures. The absence of restraint exerted on the animals, the recording of the reactions of the animals, and the possibility of using large numbers of small animals have definite advantages. The apparatus needs little care, but the basket should be inspected frequently and urine and feces should be removed to prevent short circuit. The air transmission system must of course be leakproof and should be adjusted to optimal sensitivity.

SUMMARY

A new method for testing analgesic drugs in mice is described. The method permits the application of an intermittent and increasing stimulus to mice without restriction of their movements and obtains direct records of their reactions.

REFERENCES

1. Macht, D. I., and Macht, M. B.: Quantitative Studies on Pain Threshold After Administration of Various Drugs, J. Am. Pharm. A. (Scient. Ed.) 29: 193, 1940.
2. D'Amour, F. E., and Smith, D. L.: A Method for Determining Loss of Pain Sensation, J. Pharmacol. & Exper. Therap. 72: 74, 1941.
3. Andrews, H. D., and Workman, W.: Pain Threshold Measurements in the Dog, J. Pharmacol. & Exper. Therap. 73: 99, 1941.
4. Hardy, J. D., Wolff, H. G., and Goodell, H.: Studies on Pain. A New Method for Measuring Pain Threshold: Observations on Spatial Summation of Pain, J. Clin. Investigation 19: 649, 1940.
5. Straub, W. A., and Tirendl, E.: Über die Messung analgetischsedativer Wirkungen am Tier und die Übertragbarkeit der Messungsresultate auf den Menschen, Arch. f. exper. Path. u. Pharmakol. 195: 481, 1940.
6. Forst, A. W.: Morphin und Dihydrodesoxymorphin, verglichen mittels einer neuen Methode der Motilitätsmessungen, Arch. f. exper. Path. u. Pharmakol. 192: 257, 1939.
7. Smith, D. L., D'Amour, M. C., and D'Amour, F. E.: Analgesic Properties of Certain Drugs and Drug Combinations, J. Pharmacol. & Exper. Therap. 77: 184, 1943.

CHRONIC INDURATIVE PNEUMONIA RESULTING FROM CARDIOSPASM*

A Case With Nonpathogenic Acid-fast Bacilli in the Sputum

Frederick C. Warring, Jr., M.D., and Arnold B. Rilance, M.D., Shelton, Conn.

PNEUMONITIS complicating cardiospasm does not appear to be a very common condition. Isolated cases have been reported.[1, 2] On the other hand, Sturtevant,[3] in an article reviewing the literature on cardiospasm, fails to mention pulmonary complications, apparently because of their rarity. Even more uncommon have been reports of the isolation of nonpathogenic acid-fast organisms from the sputum in this condition. Baldwin[4] has recently reported such a case, and a similar one has been described by Cummins and Williams.[5] These two cases are apparently the only ones to be found in the literature in which nonpathogenic acid-fast bacilli have been found in the sputum in this condition. A third case has lately come under our observation.

CASE HISTORY

G. H., a white female, single, aged 65 years, a bookkeeper, was admitted to the Laurel Heights State Tuberculosis Sanatorium on December 8, 1941. There was no history of exposure to tuberculosis.

Past History.—In 1922 the patient began to have difficulty in swallowing, with regurgitation of small amounts of food, but not of whole meals. There was no history of having swallowed any irritants. At that time she manifested mild jaundice, and her gall bladder, containing stones, was removed. Further studies while she was in the hospital revealed moderate cardiospasm. No dilatation was then felt to be necessary.

During the past twenty years she has been troubled by this regurgitation for periods varying in length from a few hours to three days, and then would be entirely free from this symptom for intervals up to several months. She denied aspirating vomitus at any time.

Present Illness.—Gradually, over the past year and a half, there has been a loss of 16 pounds in weight. This was probably because she ate only small amounts of food in an effort to ward off regurgitation. Seven weeks before admission the patient developed weakness, fever, and night sweats. She also began to cough, and she raised some mucoid sputum which was not foul but did contain a few food particles. On bed rest at home she failed to improve and came to Laurel Heights on December 8, 1941.

On admission her general condition was poor. Her best previous weight had been 136 pounds, but she was now down to 87. Stereoroentgenograms of the chest, taken on December 9, 1941, showed mottled densities throughout the lung field on the right, with consolidation from the level of the third to the seventh vertebral spines. On the left, there were conglomerate patches in the lower lobe (Fig. 1.). Physical examination was noncontributory, except for the lungs. There were signs of infiltration, with medium and fine râles, over the areas of disease seen in the x-ray.

The patient had asymptomatic *Bacillus coli* bacilluria, with white blood cells, but no other urinary abnormalities. Hemoglobin was 11.5 Gm. (74 per cent); red blood cells, 3.85; white blood cells, 16,500, with polymorphonuclear cells 85 per cent, lymphocytes 9 per cent,

*From the Laurel Heights State Tuberculosis Sanatorium.
Received for publication, March 10, 1943.

monocytes 5 per cent, and eosinophils 1 per cent. Sedimentation rate (Cutler) was 27.0 and 28.5 mm. Kline diagnostic test was negative. A tuberculin test was highly positive to the second strength of purified protein derivative.

X-rays of the stomach and intestines were negative. Barium was retained in the esophagus, however, for as long as ten hours, revealing obstruction at the cardiac orifice. The lower third of the esophagus was mildly and uniformly dilated. No diverticulae could be seen (Fig. 2).

The patient was felt to have a cardiospasm of twenty years' duration. By unconsciously aspirating food, probably during sleep, she had developed a chronic, bilateral induration pneumonia.

Two specimens of sputum, raised from the lungs, contained large numbers of acid-fast bacilli. Morphologically these were very similar to tubercle bacilli. The working diagnosis of aspiration pneumonia led to further studies which demonstrated the organisms to be nonpathogenic, acid-fast saprophytes.

Fig. 1.—X-ray of the chest on admission 12/8/41.

On bed rest the patient ran a continuous fever from 100° to 101° F. Her pulse ranged from 75 to 85. During her two months' stay in the Sanatorium there was no significant change in the appearance of serial x-rays of the lungs. Occasional regurgitation persisted, but she gained a little in weight and strength. On February 12, 1942, she was discharged, to receive treatment elsewhere for the cardiospasm.

In March, 1942, Miss H. was treated by Doctor Norton Canfield at the New Haven Hospital. Dilatation of the lower end of the esophagus was accomplished by means of the McKenzie dilator. After two weeks, good esophageal function was obtained. Since that time she has been able to swallow and retain both liquids and solid foods without difficulty.

The patient returned to our Out-patient Department for a check-up on October 9, 1942. Her general condition was much improved and she had gained about twenty pounds. She felt

stronger, her fever had disappeared, and she had practically no cough or expectoration. Chest roentgenograms showed pronounced clearing of the densities in both lungs, although a good deal of chronic, fibrous infiltration remained (Fig. 3). No acid-fast organisms could be demonstrated in the sputum by smear or culture at this visit.

Fig. 2.—X-ray taken 10 hours after barium meal showing cardiospasm.

THE ORGANISM

On admission, in December, 1941, acid-fast rods were found in good numbers in the sputum on direct smear. Morphologically they closely resembled tubercle bacilli, although a few acid-fast coccoid bodies were also present. The organism grew well in Hamburg broth in twenty-four hours. On dextrose agar, after forty-eight hours, the colonies were smooth and had a slight cream color, taking on an orange tinge as the culture aged. No carbohydrates were fermented. After several subcultures the morphology changed markedly. The rods disappeared, leaving mostly acid-resistant, deep-staining, coccoid bodies, with some fine, faintly staining filamentous forms. Guinea pigs inoculated with the bacteria did not develop tuberculosis, nor did they become sensitized to old tuberculin.

Doctor Friend Lee Mickle of the Connecticut State Laboratory and Mr. William Steenkin, Jr., at the Research and Clinical Laboratory of Trudeau, New York, studied these cultures with us. It was agreed that the organism was a nonpathogenic acid-fast saprophyte.

Doctor Edward R. Baldwin of Saranac Lake, New York, made a tuberculin from cultures of this saprophyte. Skin-tested with 0.1 mg. of this autogenous tuberculin, the patient reacted with an area of erythema measuring 3.5 × 4.0 cm. and marked induration. An intradermal control test, using 0.1 mg. of old tuberculin, produced an area of erythema 4.5 × 6.5 cm. with pronounced induration.

Twelve other patients with proved, active pulmonary tuberculosis were skin-tested, using 0.1 mg. of the autogenous tuberculin in six patients and 1.0 mg. in the remaining six. Only two patients, both of whom received the 1.0 mg. dose of the autogenous tuberculin, showed a mild, but typical positive reaction, with erythema and induration. All twelve cases reacted strongly to 0.1 mg. of regular old tuberculin.

Fig. 3.—X-ray of the chest taken on 10/9/42, seven months after treatment of cardiospasm by dilatation. Moderate clearing of infiltration in both lungs.

These studies showed that the patient had acquired a marked degree of sensitivity to the nonpathogen in her sputum. Almost complete lack of reaction in the tuberculous controls demonstrated that this could not be explained adequately by cross sensitivity.

COMMENT

This report of another case of aspiration pneumonitis, secondary to cardiospasm, in which acid-fast bacilli have been found in the sputum, would suggest

a relationship that is more than accidental. As proposed by Baldwin,[4] a logical explanation for the source of the nonpathogenic acid-fast bacilli is to be found in the aspiration of food particles from the esophagus into the lungs. There the organism may become established and occasionally make its appearance in the sputum. Similar nonpathogenic acid-fast organisms have been cultured from many raw fruits and vegetables.[4]

The question arises whether or not this acid-fast saprophyte had developed some slight degree of pathogenicity for this particular patient. Probably not, since it did not seem to influence the course of the aspiration pneumonia and did not produce disease in guinea pigs. On the other hand, it appears that the bacteria did not merely lodge inertly in the bronchial tree, but established themselves as facultative parasites. This assumption is supported by the development of the marked sensitivity, absent in controls, to the tuberculin prepared from the acid-fast bacilli in her sputum.

The finding of these acid-fast rods in the sputum on direct smear is of definite interest. The differentiation between them and tubercle bacilli on the basis of their morphologic characteristics would be extremely difficult. Thorough bacteriologic studies should be performed on any patient suspected of having an aspiration pneumonitis, in which acid-fast organisms are found in the sputum. Otherwise a mistaken diagnosis of pulmonary tuberculosis may readily be made.

The short length of time during which the acid-fast bacilli were found in the sputum and the rapidity with which they changed to coccoid forms may explain why more of these cases have not been reported. Apparently only under certain favorable cultural environments do they retain their acid-fast bacillary form. This is true both in vivo and in vitro. The rapidity with which the organism multiplies (twenty-four to forty-eight hours) on most laboratory media is one means of differentiating them from tubercle bacilli.

SUMMARY

A case of aspiration pneumonitis resulting from cardiospasm is reported, in which saprophytic acid-fast bacilli were found by direct smear in the sputum. Morphologically these bacteria resembled tubercle bacilli. The bacteriologic characteristics of the organism are described.

REFERENCES

1. Vinson, P. P.: Cardiospasm Complicated by Pulmonary Abscess, Am. J. Surg. 2: 359, 1927.
2. Sampson, D. A.: Lung Abscess Due to Oesophageal Overflow, New England J. Med. 229: 982, 1938.
3. Sturtevant, M.: Cardiospasm, Arch. Int. Med. 51: 74, 1933.
4. Baldwin, E. R.: Non-pathogenic Acid-fast Bacilli, Am. Rev. Tuberc. 45: 756, 1942.
5. Cummins, S. L., and Williams, E. M.: "Acid-fast" Other Than Koch's Bacillus Cultivated From Sputum. Tubercle 15: 49, 1933.

CLINICAL CHEMISTRY

STUDIES IN PROTHROMBIN: V. ARTERIAL AND VENOUS PLASMA PROTHROMBIN TIME IN MAN*

SHEPARD SHAPIRO, M.D., NEW YORK, N. Y.

THE liver is essential for the elaboration of prothrombin.[1] Although other tissues seem not to be involved directly in the process, nevertheless, recent observations suggest that under certain conditions additional mechanisms might contribute toward the maintenance of prothrombin at a level (or activity) higher than normal. Thus, hyperprothrombinemia has been demonstrated in (a) acute thrombophlebitis and the initial stage of thrombo-embolization[2, 3] and (b) multiple myeloma.[3]

It has been noted by Andrus, Lord, and Kuner[4] in 17 out of 20 experiments on dogs that blood obtained from the left ventricle exhibited less prothrombin activity than that removed from the right ventricle. The average difference was 10.6 per cent, determined by the two-stage method of Warner, Brinkhous, and Smith.[5] The visceral and peripheral arterial and venous bloods showed no significant difference.

These observations led to the present investigation to determine what is the relationship between the peripheral arterial and venous blood plasma prothrombin time in man.

The prothrombin times of arterial and venous plasma of twenty-six patients were studied. In 11 of these, the venous plasma prothrombin times were normal; in the remaining 15, the prothrombin times were prolonged as a result of existing liver disease, nutritional (vitamin K?) deficiency, or therapeutic agents which decrease the level (or activity) of prothrombin (salicylates, sulfanyl compounds, Dicumarol).

Method of Prothrombin Assay.—The procedure used to estimate the prothrombin time is based upon the single-stage method of Quick[6] and includes estimation of the prothrombin time of whole and diluted (12.5 per cent) plasma. The rationale, clinical and experimental applications have been set forth in previous communications.[2, 3, 7-11] The greater sensitivity of 12.5 per cent plasma over that of whole plasma also has been demonstrated.[12, 13]

Thromboplastin prepared from fresh rabbit lung was used. The established normal standard for 12.5 per cent (venous) plasma was 39.5 seconds (Standard deviation = ±2.5). All estimations were done in duplicate.

The specimens of venous blood were obtained in the usual manner of venipuncture. The arterial blood samples were taken from the radial artery. The cutaneous area of puncture was first anesthetized with 1 per cent novocain.

---

*From the Department of Medicine, New York University, and the Third (New York University) Division, Goldwater Memorial Hospital, Welfare Island, New York.

Received for publication, June 24, 1943.

Plasma was obtained by the addition of 4.5 c.c. of blood to 0.5 c.c. 1/10 molar sodium oxalate and centrifugation.

Results.—More than 100 estimations of the prothrombin time of arterial and venous plasma obtained from the 26 individuals were made. In three patients serial estimations were made daily for one week. In every instance the difference between the arterial and venous plasma prothrombin time was found to be within the limits of statistical variation. In three cases the differences were on the first examination slightly in excess of these limits. Repetition of the determinations on subsequent days, however, revealed no significant difference between the respective arterial and venous plasma prothrombin time.

Discussion.—In normal individuals the prothrombin time of arterial blood was a trifle greater than that of venous blood. The magnitude of the difference was in almost every instance less than three seconds and hence within normal variation and consequently to be considered as statistically not significant.

The arterial and venous plasma prothrombin time in four cases of Laennec's cirrhosis, although moderately or markedly prolonged,* were almost identical in each instance.

Prothrombinopenia, whether induced by agents such as Dicumarol,[2, 11] salicylate[12, 13] or sulfanyl compounds,[15, 16] or occurring spontaneously as a result of chronic debility (nutritional [vitamin K?] deficiency), does not alter the relationship which appears normally to exist between the prothrombin time of arterial and venous plasma.

It is apparent, therefore, that in man (as in dogs[4]) no significant difference between arterial and venous plasma prothrombin time is demonstrable. Should such a variation arise it would be distinctly abnormal. It might conceivably occur as a result of an unusual interchange within the pulmonary system or at the peripheral capillaries.

It is here established that for practical purposes arterial or venous blood may be used interchangeably for estimation of the prothrombin time.

Summary.—The greater sensitivity of diluted plasma as compared with whole plasma for estimation of prothrombin time is emphasized.

The prothrombin time of peripheral arterial plasma is generally a trifle in excess of that of venous plasma. The difference, however, is within the range of statistical variation and consequently not significant.

The findings herein recorded indicate that when prothrombinopenia is induced by agents such as Dicumarol, salicylate, or sulfanyl compounds, the arterial and venous plasmas are equally affected. This is also true of prothrombinopenia arising spontaneously in consequence of debilitating disease. Hence, the normally existing relationship between arterial and venous plasma is unaffected by the development of prothrombinopenia.

Arterial and venous blood may be used interchangeably for estimation of prothrombin time.

The work was conducted in part at the Home of the Daughters of Israel, New York City, through the generous cooperation of Dr. Julius Raab.

The author wishes to thank the following physicians for their assistance in obtaining blood samples. Dr. Robert C. Murphy, Jr. (St. Lukes Hospital); Dr. Sylvan M. Stillman (Bellevue Hospital).

Dicumarol was supplied by Dr. R. K. Richards of Abbott Laboratories, North Chicago, Ill.

Miss Frances Kaufman gave technical assistance.

---

*Moderate prolongation: between 1½ and twice normal. Marked prolongation: greater than twice normal.[14]

REFERENCES

1. Smith, H. P., Warner, E. D., and Brinkhous, K. M.: Prothrombin Deficiency and the Bleeding Tendency in Liver Injury (Chloroform Intoxication), J. Exper. Med. 66: 801, 1937.
2. Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A.: Prothrombin Estimation: A Procedure and Clinical Interpretations, Proc. Soc. Exper. Biol. & Med. 50: 85, 1942.
3. Shapiro, S., Ross, V., and Moore, D. H.: A Viscous Protein Obtained in Large Amount From the Serum of a Patient With Multiple Myeloma, J. Clin. Investigation 22: 137, 1943.
4. Andrus, W. De W., Lord, J. W., Jr., and Kauer, J. T.: Studies on the Fate of Plasma Prothrombin, Science 91: 48, 1940.
5. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: Quantitative Study on Blood Clotting: Prothrombin Fluctuations Under Experimental Conditions, Am. J. Physiol. 114: 667, 1936.
6. Quick, A. J., Stanley-Brown, M., and Bancroft, F. W.: Study of the Coagulation Defect in Hemophilia and Jaundice, Am. J. M. Sc. 190: 501, 1935.
7. Shapiro, S., Sherwin, B., and Gardiner, H.: The Platelet Count and the Prothrombin Time After Surgical Operations. A Simple Method for Detecting Reductions and Elevations of the Prothrombin Concentration (or Activity) of the Blood Plasma, Ann. Surg. 116: 175, 1942.
8. Shapiro, S., and Sherwin, B.: Studies in Thrombo-Embolization II: Observations on the Use of Dicumarol (3, 3' Methylenebis (4-Hydroxycoumarin)) in Embolization. Report of Five Cases, New York State J. Med. 43: 45, 1943.
9. Shapiro, S.: Symposium on Dicumarol (Discussion), J. A. M. A. 120: 1024, 1942.
10. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease; the Bioassay of Hemorrhagic Concentrates by Following the Prothrombin Level in the Plasma of Rabbit Blood, J. Biol. Chem. 138: 1, 1941.
11. Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease VII: The Effect of 3, 3' Methylenebis (4-Hydroxycoumarin) on the Prothrombin Time of the Plasma of Various Animals, J. Biol. Chem. 142: 941, 1942.
12. Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D.: Studies on the Hemorrhagic Sweet Clover Disease XI: Hypoprothrombinemia in the Rat Induced by Salicylic Acid, J. Biol. Chem. 147: 463, 1943.
13. Shapiro, S., Redish, M. H., and Campbell, H. A.: The Prothrombinopenic Effect of Salicylates in Man, Proc. Soc. Exp. Biol. & Med. 53: 251, 1943.
14. Shapiro, S., Redish, M. H., and Campbell, H. A.: Studies on Prothrombin II: The Effects of a Single Small Dose of Dicumarol (3, 3' Methylenebis (4-hydroxycoumarin)) in Liver Disease, Am. J. M. Sc. 205: 808, 1943.
15. Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P.: The Effect of Sulfaguanidine on Rat Growth and Plasma Prothrombin, J. Biol. Chem. 145: 137, 1942.
16. Kapnick, I., Stewart, J. D., and Lyons, C.: Plasma Prothrombin and Liver Function During Sulfonamide Therapy, New England J. Med. 227: 941, 1942.

LABORATORY METHODS

GENERAL

USE OF TERTIARY BUTYL ALCOHOL IN BACTERIOLOGIC STAINING PROCEDURES*

Parker R. Beamer, Ph.D., M.D., and Robert E. Stowell, M.D., St. Louis, Mo.

Bacteriologists and clinical pathologists may desire satisfactory substitutes for certain reagents which are becoming increasingly expensive and difficult to obtain.

Tertiary butyl alcohol was introduced into microtechnical procedures by Johansen,[1] who subsequently developed methods for its use as a dehydrating agent in plant microtechnique.[2-4] Stowell[5, 6] found this reagent satisfactory for dehydration of a large variety of mammalian tissues and recommended its use in place of ethyl alcohol to help conserve reagents which are vital to our war industries.[7]

Various solvents such as methyl, ethyl, and isopropyl alcohols and acetone have been used as decolorizing agents in making Gram stains of bacterial films. In the present study over five hundred preparations of pure cultures, mixed suspensions, unknown organisms isolated at autopsy, urethral pus, and tuberculous sputum were stained. Except for sputum, the films were stained by the routine Gram staining procedure used in this laboratory and also by a procedure substituting tertiary butyl alcohol for ethyl alcohol as a stain solvent and decolorizing reagent; likewise, in the destaining of nonacid-fast organisms in sputum, tertiary butyl alcohol was substituted for ethyl alcohol.

EXPERIMENTAL

Preparation of Solutions.—The primary Gram stain was prepared by dissolving 2 Gm. of crystal violet (or gentian violet, Coleman-Bell) in 20 c.c. of tertiary butyl alcohol. This was then mixed with 80 c.c. of a 1 per cent aqueous solution of ammonium oxalate and filtered. Gram's iodine solution (0.33 per cent iodine, 0.67 per cent potassium iodide in distilled water with a small lump of calcium carbonate added to stabilize the solution) was used as the mordant. Ethyl alcohol (95 per cent) or tertiary butyl alcohol was used to destain the Gram negative organisms. The counterstain was a 2.5 per cent aqueous solution of safranine.

Carbolfuchsin solution employed in a modified Ziehl-Neelsen technique was prepared by dissolving 0.3 Gm. of basic fuchsin in 10 c.c. of tertiary butyl alco-

*From the Department of Pathology, Washington University School of Medicine, and The Barnard Free Skin and Cancer Hospital, St. Louis, Missouri.

Received for publication, November 14, 1942.

hol and mixing this with 90 c.c. of a 5 per cent aqueous solution of phenol. A 5 per cent solution of nitric acid in tertiary butyl alcohol or in ethyl alcohol (95 per cent) was used for decolorizing.

Procedures and Results.—In order to determine the proper time relations when tertiary butyl alcohol was used as a decolorizing agent, *Staphylococcus aureus* and *Escherichia coli* were selected as test organisms. Several bacterial films made in the usual manner were stained by the modification of Gram's procedure used satisfactorily in this laboratory for routine work (crystal violet, iodine solution, 95 per cent ethyl alcohol, and safranine applied in order, ten to fifteen seconds each, followed by washing in tap water). Similar films were prepared and stained in order to test the action of various concentrations of tertiary butyl alcohol as a destaining agent. Dilutions of tertiary butyl alcohol were prepared, and each was used in several series of stains in which the time of application of the primary stain, the iodine solution, or the decolorizing agent was varied. For example, in one series the primary stain and iodine solution were applied for a constant time and the period of destaining was altered for individual slides. Each slide was compared with one or more stained by the routine method using ethyl alcohol. When a satisfactory time for decolorizing was determined, other series were studied in which the time of primary staining or application of iodine solution was altered. These slides, likewise, were compared with routine stains. The series were repeated with each of the aqueous dilutions of tertiary butyl alcohol. In general, the periods of time (ten to sixty seconds) during which the primary stain or the iodine solution was applied made little difference in the results.

With 10, 20, and 30 per cent solutions of tertiary butyl alcohol the results were variable and unsatisfactory. When applied as long as five minutes, 50 per cent or less of the Gram negative forms were properly decolorized. Films which were decolorized with 40, 50, or 60 per cent tertiary butyl alcohol showed better differentiation between Gram negative and positive organisms, but variable numbers of the Gram negative forms were not destained unless the tertiary butyl alcohol was applied for two minutes or longer. Under these conditions, however, the Gram positive organisms in the thinner portions of the films were slightly decolorized. This was true especially if the iodine solution had been prepared several weeks previously.

After further studies it was found that tertiary butyl alcohol in concentrations of 60, 70, 80, or 90 per cent was satisfactory for destaining. Dependent on certain factors such as thickness of the film, the time of destaining, and the stability of the iodine solution, Gram negative organisms were properly decolorized and differentiation was good. The most satisfactory results were obtained when thin bacterial films were stained in the following manner:

1. Crystal violet solution, ten seconds; wash in tap water.
2. Iodine solution, ten seconds; wash in tap water and shake slide free of water.
3. Eighty per cent tertiary butyl alcohol, sixty to ninety seconds; wash in tap water.
4. Safranine solution, ten to fifteen seconds; wash and dry.

If thin, even films were prepared, 60 per cent and 70 per cent concentrations of tertiary butyl alcohol differentiated Gram negative forms well but less satisfactorily than did the procedure outlined above. Stains prepared according to this method were compared with those made by the routine procedure (using 95 per cent ethyl alcohol as a decolorizing agent) in identifying organisms isolated from nearly one hundred patients at autopsy. During the course of these studies the following organisms were cultured and satisfactorily stained by the method outlined: *Proteus vulgaris, Serratia marcescens, Staphylococcus aureus* and *albus, Escherichia coli, Alkaligenes faecalis,* streptococci, *Actinomyces hominis,* diphtheroids, *Sarcina lutea, Neisseria catarrhalis,* unidentified sporulating rods, *Pseudomonas aeruginosa,* and two type cultures of Salmonella.

In applying this method to several smears of urethral pus from different patients with acute gonorrhea, it was observed that, except with very thin smears, the time of destaining should be extended to two or three minutes. As other workers have indicated, preparations were satisfactory when pus cells were stained well with the counterstain.

A 5 per cent solution of nitric acid in 50 to 80 per cent tertiary butyl alcohol was satisfactory for decolorizing nonacid-fast bacteria in specimens of tuberculous sputum. Excellent differentiation was obtained by the application of steaming carbolfuchsin for five minutes, followed by destaining for one to two minutes before applying the counterstain.

DISCUSSION

The procedure outlined in this study is not proposed to supplant methods now used in preparing Gram stains, but rather to suggest a method which may be employed satisfactorily in the event that reagents now in common use become too expensive or difficult to obtain. Tertiary butyl alcohol, therefore, may be of importance in bacteriologic staining procedures, since it yields satisfactory results and it is a surplus by-product in the oil industry. According to sales agents[8] of the manufacturer, the supply of tertiary butyl alcohol is plentiful, and it may be obtained without any difficulty. This has been confirmed by the War Production Board.[9] In contrast, the supply of normal and secondary butyl alcohols is limited owing to their important uses in war industries. In the United States most of the tertiary butyl alcohol is marketed as one anhydrous grade produced by one oil company. Retail prices, however, show considerable variation, some firms asking about ten times the wholesale cost. On the basis of comparative prices of 55-gallon drum lots, tertiary butyl alcohol, which is tax-free, is only slightly more expensive than the average price of 95 per cent ethyl alcohol without the added tax.

Pure tertiary butyl alcohol, $(CH_3)_3$—C—OH, although said to be three times as toxic as ethyl alcohol, should be safe for use in the laboratory if ordinary precautions are taken to avoid prolonged exposure to high concentrations of the alcoholic vapors. The compound melts at 23 to 25.6° C., and as traces of water are taken up the melting point is lowered. When the melting point is above 23° C., at least 99.4 per cent of the reagent is present. If the room temperature is below the melting point of the alcohol, the stock bottle may be left in a warm place such as the top of an oven or near a radiator. Tertiary butyl

alcohol is miscible with most reagents in common use in the laboratory, including water, ethyl alcohol, ether, xylene, benzene, and chloroform.

SUMMARY

Tertiary butyl alcohol, a readily available, reasonably priced reagent, is satisfactory as a decolorizing agent in modifications of the Gram and Ziehl-Neelsen staining procedures. Should commonly used reagents become too expensive or unobtainable, tertiary butyl alcoohl may be used in these methods.

REFERENCES

1. Johansen, D. A.: Dehydration and Infiltration, Science 82: 253, 1935.
2. Johansen, D. A.: Tertiary Butyl Alcohol Methods, El Palo Alto News 1: 1, 5, 13, 21, 1937.
3. Johansen, D. A.: Tertiary Butyl Alcohol Methods, El Palo Alto News 2: 1, 1938.
4. Johansen, D. A.: Plant Microtechnique, New York, 1940, McGraw-Hill Book Company, p. 523.
5. Stowell, R. E.: Effect on Tissue Volume of Various Methods of Fixation, Dehydration, and Embedding, Stain Technol. 16: 67, 1941.
6. Stowell, R. E.: The Use of Tertiary Butyl Alcohol in Micro-technique, Science 96: 165, 1942.
7. Stowell, R. E.: Tertiary Butyl Alcohol as a Microtechnical Reagent, J. Tech. Methods 22: 71, 1942.
8. R. W. Greeff and Co., Inc., 10 Rockefeller Plaza, N. Y.: Personal communication.
9. War Production Board (Alcohol and Solvents Section), Washington, D. C.: Personal communication.

A NEW AND RAPID STAINING METHOD FOR GRAM-POSITIVE AND GRAM-NEGATIVE ORGANISMS IN FROZEN AND PARAFFIN SECTIONS

Aram A. Krajian, Sc.D., Los Angeles, Calif.

Christian Gram[1] in 1884 devised his method for the demonstration of bacteria in tissue sections. Weigert's[2] modification of Gram's method has been useful for the demonstration of fibrin and bacteria and is still widely used, but both methods demonstrate only gram-positive organisms.

Since then, numerous attempts have been made to demonstrate both gram-positive and gram-negative organisms in paraffin sections of tissues that had been fixed in Zenker's solution or in formaldehyde solution, and methods accomplishing this purpose have been reported by MacCallum,[3] Lyon,[4] Lillie,[5] Brown and Brenn,[6] Rudnikoff and Stawsky,[7] Glynn,[8] and others.

In these modifications, the staining solutions as devised by Gram remained practically unchanged. No attempt has been made to devise a new method by the use of some other dye or chemical, and no recommendations have been made as to the applicability to frozen sections.

The solutions used by Gram and used in the modifications of his method are: gentian violet, methyl violet, and crystal violet, as gram-positive stains; Gram's solution and compound solution of iodine, U.S.P. (Lugol's solution), as

Received for publication, November 22, 1942.

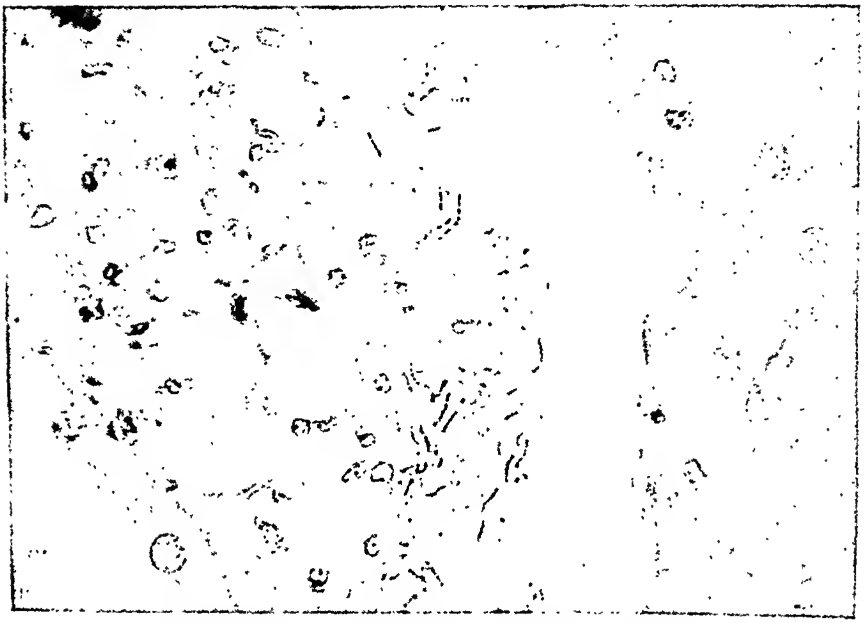


Fig. 1.—Streptococcus in kidney, stained blue; ×1620.

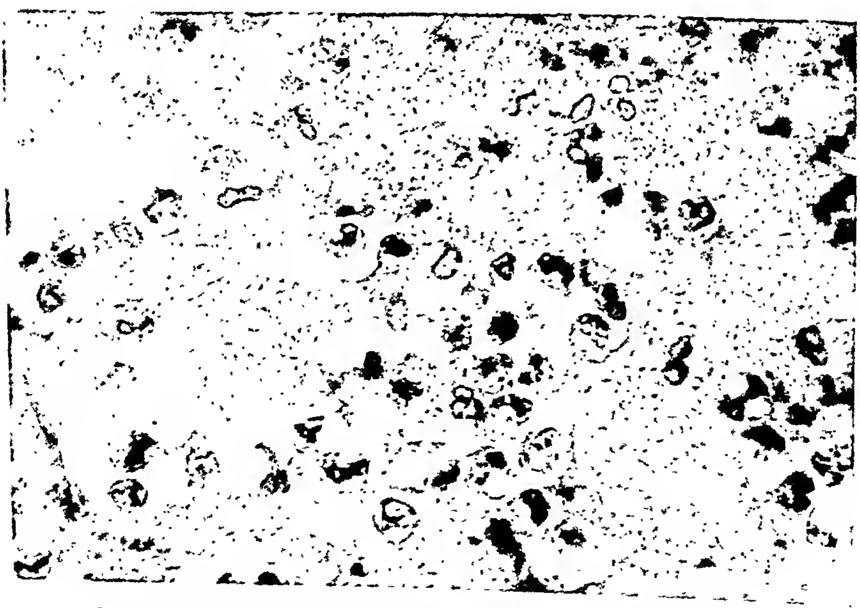


Fig. 2.—Friedländer's bacilli in pneumonia, stained red; ×1620.

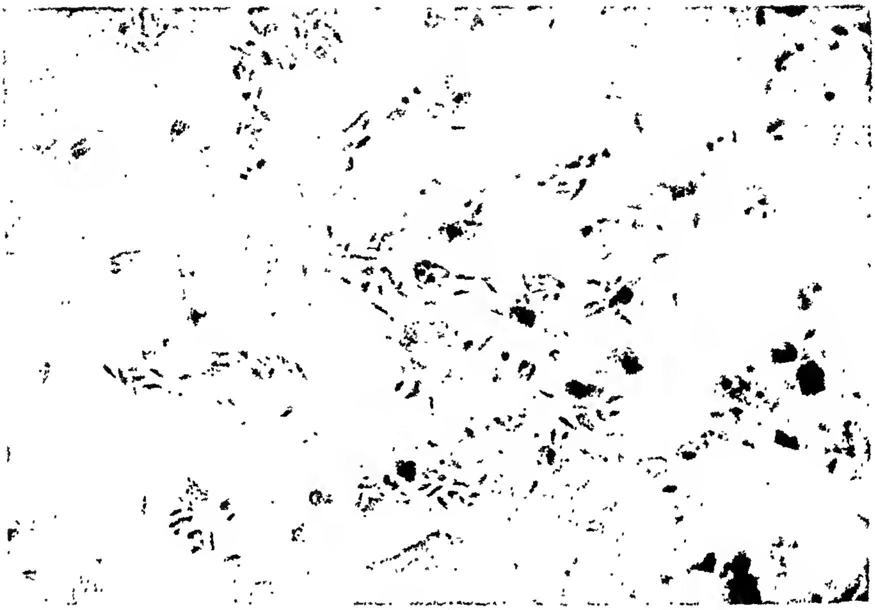


Fig. 3.—Sporotrichum in testicle, stained blue; ×1620.

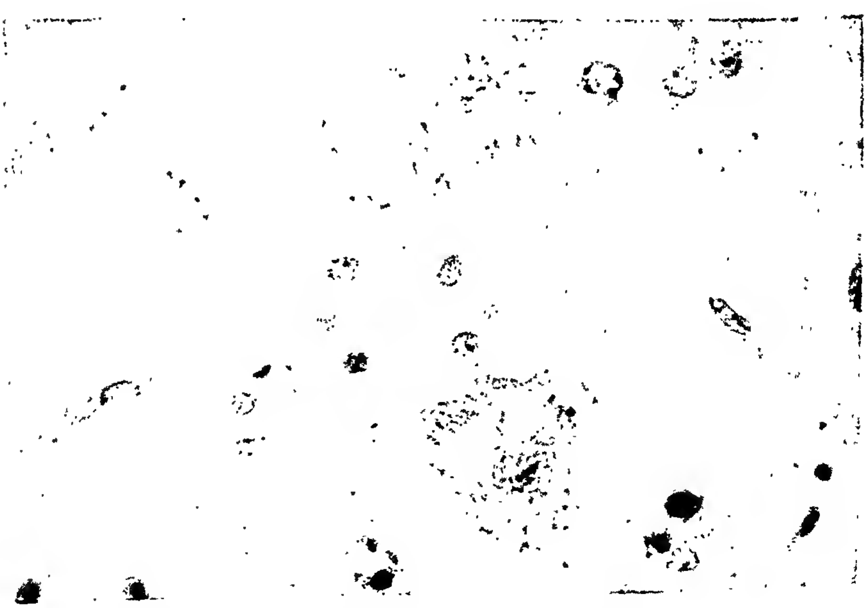


Fig. 4.—Colon bacilli in kidney tubules, stained red; ×1620.

differentiators; rosaniline hydrochloride, basic fuchsin, dilute carbolfuchsin, and safranine as gram-negative stains; and acetone, alcohol, ether, and aniline oil-xylene as decolorizers.

The need for rapid diagnosis led me to investigate the usefulness of a different dye and different solutions. A new and simple method was devised which is easily applied to frozen and paraffin sections of formaldehyde fixed tissue and which gives satisfactory simultaneous differential staining of gram-positive and gram-negative organisms. For example, four different sections (frozen or paraffin) mounted on the same slide, one of each of a kidney containing monilia, a lung with Friedländer's bacilli, a testicle containing sporotrichum, and a kidney with colon bacilli, were stained, with successful differentiation of the organisms by the method described. The technique has also been used on tissues containing streptococci, Welch bacilli, influenza bacilli, actinomyces, meningococci, gonococci, diphtheria bacilli, and Negri bodies. Similar results are obtained in another method recently described by me,[9] but the time and material required has been greatly decreased in the present technique.

In this new method, the solutions used are: methylene blue as a gram-positive stain, fuchsin-creosote as a gram-negative stain, creosote-xylene as a differentiator.

THE FROZEN SECTION METHOD

1. Prepare mounted frozen sections at 7 to 10 microns in the usual manner.
2. Stain for three minutes with alkaline methylene blue (Loeffler's).
3. Wash in tap water and dehydrate rapidly with three applications of anhydrous isopropanol or absolute ethyl alcohol.
4. Differentiate rapidly with creosote-xylene (1 part creosote plus 2 parts xylene), agitating slide constantly for five to ten seconds.
5. Pour off and apply creosote-fuchsin (2.5 c.c. of a 6 per cent alcoholic basic fuchsin added to 50 c.c. of creosote-xylene), agitating slide about 15 to 20 times, changing the solution once.
6. Blot and apply creosote-xylene 2 or 3 times, agitating the slide constantly for even decolorization or until most of the excess red color leaves the section.
7. Blot, clear for two minutes in xylene and mount in gum dammar.

FOR PARAFFIN SECTIONS

Deparaffinize section with two applications of xylene and two applications of absolute alcohol or isopropanol; bring down to tap water and then proceed with the method described.

With the use of this method, nuclei are red, gram-positive organisms blue, gram-negative organisms red, devitalized gram-positive organisms red, monilias and actinomyces blue, Negri bodies bright red with bluish chromatin bodies, and fibrin sometimes blue and sometimes red.

All the staining solutions are stable.

SUMMARY

A new, simple, and rapid method for the demonstration of gram-positive and gram-negative organisms in frozen and paraffin sections of formaldehyde fixed tissues is presented. It has the particular advantage of demonstrating the

devitalized forms of gram-positive organisms. The staining solutions used are: methylene blue, fuchsin-creosote, creosote-xylene.

REFERENCES

1. Gram, C.: Ueber die isolirte Färbung der Schizomyceten in Schnitt—und Trockenpräparaten, Fortschr. d. Med. 2: 185, 1884.
2. Weigert, C.: Ueber eine neue Methode zur Färbung von Fibrin und von Micro-organismen, Fortschr. d. Med. 5: 228, 1887.
3. MacCallum, W. G.: A Stain for Influenza Bacilli in Tissue—A Combination of Goodpasture's and Weigert's Stains, J. A. M. A. 72: 193, 1919.
4. Lyon, M. W., Jr.: Acetone as a Decolorizer in Gram's Staining Method, J. A. M. A. 75: 1017, 1920.
5. Lillie, R. D.: The Gram Stain—A Quick Method for Staining Gram-Positive Organisms in Tissues, Arch. Path. 5: 828, 1928.
6. Brown, J. Howard, and Brenn, Lena: A Method for the Differential Staining of Gram Positive and Gram Negative Bacteria in Tissue Sections, Bulletin Johns Hopkins Hospital 48: 69, 1931.
7. Rudnikoff, I., and Slawsky, Helen: Modified Method for Staining Gram Positive and Gram Negative Organisms in Tissue Section, Arch. Path. 19: 513, 1935.
8. Glynn, John H.: The Application of the Gram Stain to Paraffin Sections, Arch. Path. 20: 896, 1935.
9. Krajian, Aram A.: A New Staining Method for Gram Positive and Gram Negative Organisms in Frozen Section, Arch. Path. 32: 825, 1941.

LITMUS PAPER DISPENSER*

E. E. Beohm, A.B., Philippi, W. Va.

IN USING litmus paper to test the acidity of urine the usual method of taking a strip from a vial and tearing off a piece tends to be wasteful of paper and is otherwise unsatisfactory.

Fig. 1.

The use of vials filled with cut squares to be removed by the fingers likewise has its disadvantages in that the fingers must be dry; else some of the paper may be ruined, etc.

*From The Myers Clinic Laboratory, Philippi, W. Va.
Received for publication, November 22, 1942.

To eliminate these difficulties we have devised a litmus paper dispenser, which has proved to be a distinct aid in our laboratory. A Petri dish is used to hold squares of litmus paper (best cut from one strip at a time so that they will not tend to stick together). The dish is divided into two compartments for red and blue paper by a piece of 7 mm. glass rod, cemented to the glass by household cement. A pair of tweezers is used to pick up the squares of paper.

Fig. 1 is a photograph of the dispenser.

THE COMBINING POWER OF STAPHYLOCOCCUS TOXOID AS AN IDENTITY TEST*

LYON P. STREAN, M.Sc., Ph.D., D.D.S., MONTREAL, CANADA

MANY favorable reports on the use of staphylococcus toxoid in the treatment of localized staphylococcal infections have now appeared (Dolman,[1] Connor and McKie,[2] Whitby,[3] and Murray[4]). The control of innocuity and antigenic potency of this product is governed adequately by the regulations of the National Institute of Health relating to staphylococcus toxoid. The same regulations also require identity tests, where possible, for all biologicals.

It is the purpose of this communication to outline a procedure which has proved satisfactory over a period of several years as an identity test for staphylococcus toxoid.

The method employed for identifying staphylococcus toxoid consists essentially of its ability to combine with staphylococcus antitoxin in the presence of rabbit red blood corpuscles as an indicator. It is necessary to have staphylococcus alpha toxin of suitable hemolytic power for rabbit red cells.

PROCEDURE

The first step in the procedure is to determine the lethal dose of the toxin, and this is done in the following manner. Serial dilutions of toxin are made in ten serologic tubes so that Tube 1 contains 1 c.c. of a 1:10 dilution and Tube 10 contains 1 c.c. of a 1:100 dilution. Standard staphylococcus alpha antitoxin is added to the tubes in constant quantity so that each c.c. contains 1 International Unit. One c.c. of 4 per cent washed rabbit erythrocytes is added to each tube and the final volume is made up to 4 c.c. with physiologic saline. The tubes are incubated in a water bath for sixty minutes at 37° C. A sample titration is found in Table I.

The 50 per cent end point is observed in Tube 7, which contains staphylococcus toxin in a dilution of 1:25, and the lethal dose is considered as 0.04 c.c.

In order to establish the identity of the unknown material (staphylococcus toxoid?), the titration is altered somewhat so that the material under test is con-

*From the Research and Biological Laboratories, Ayerst, McKenna and Harrison, Ltd., Montreal.

Received for publication, December 12, 1942.

tained in the hemolytic system. One c.c. of the material may be diluted 1:2 or 1:4 and is added in constant quantity. A sample titration is found on Table II.

Thus it is observed that by the addition of the unknown material to the hemolytic system, the 50 per cent end point was moved from Tube 7 to Tube 9, proving that combination has taken place between the unknown material and staphylococcus antitoxin. The only variable between the two hemolytic systems being the unknown in the second titration; therefore, the conclusion can justifiably be drawn that the unknown material must be staphylococcus toxoid.

TABLE I

TITRATION TO DETERMINE LETHAL DOSE OF STAPHYLOCOCCUS ALPHA TOXIN

TUBE NO.	1 c.c.	2 c.c.	3 c.c.	4 c.c.	5 c.c.	6 c.c.	7 c.c.	8 c.c.	9 c.c.	10 c.c.
Staphylococcus Toxin 1:10 Dil.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Saline	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9
Staphylococcus Antitoxin 1 I.U.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rabbit red blood cells 4 per cent	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Results per cent hemolysis	100	100	100	100	100	90	50	25	0	0

TABLE II

TITRATION TO DETERMINE COMBINING POWER OF MATERIAL (STAPHYLOCOCCUS TOXOID) UNDER TEST

TUBE NO.	1 c.c.	2 c.c.	3 c.c.	4 c.c.	5 c.c.	6 c.c.	7 c.c.	8 c.c.	9 c.c.	10 c.c.
Unknown 1:4 Dil.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Staphylococcus Toxin 1:10 Dil.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Saline	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Staphylococcus Antitoxin 1 I.U.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Rabbit red blood cells 4 per cent	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Results per cent Hemolysis	100	100	100	100	100	100	100	90	50	0

CONCLUSIONS

The procedure described for the identification of staphylococcus toxoid is simple and efficient. Since the lethal dose of the staphylococcus toxin must be determined as one of the mandatory tests in the establishment of the potency of the material, no added substances are necessary in the performance of the identity test save that of the material under test.

REFERENCES

1. Dolman, C. E.: The Treatment of Localized Staphylococcic Infections With Staphylococcus Toxoid, J. A. M. A. 100: 1007, 1933.
2. Connor, J. I., and McKie, M.: Anti-haemolytic Titer of Sera After Staphylococcal Infection, Brit. J. Derm. and Syph. 46: 20, 1934.
3. Whitby, L. E. H.: Modern Treatment of Staphylococcal and Pneumococcal Infections, Lancet 227: 779, 1934.
4. Murray, D. S.: Staphylococcus Toxoid—A Clinical Trial, Lancet 228: 303, 1935.

RAPID BIOLOGICAL METHOD FOR THE APPROXIMATE DETERMINATION OF ACETYLCHOLINE AND POTASSIUM IN BODY FLUIDS*

CLARA TORDA, PH.D., M.D., NEW YORK, N. Y.

THE acetylcholine content of body fluids is usually determined by biological methods. Since potassium affects the result and its content in the body fluids varies greatly, it is necessary to differentiate its effects from those of acetylcholine. The differentiation is possible by the combined application of two or more of the methods in common use. Some of the more common methods are:

(1) Chemical determination of the acetylcholine content.
(2) Chemical determination of the potassium content.
(3) Comparison of the effect of various dilutions of the body fluid on a sensitive tissue.
(4) Precipitation of potassium by permutit (Lánczos.[1]).
(5) Comparison of the effect of the unknown body fluid on the biological test objects before and after eserinization.
(6) Comparison of the effect of the unknown body fluid on the biological test objects before and after atropinization.

Such a combination, while giving reliable results, requires much time.

This paper describes a rapid method for the approximate determination of acetylcholine and potassium. The effects of the procedure are fully reversible, making a check possible. The method is based on the property of calcium to counteract the effect of potassium. The rectus abdominis muscle of the frog was used as the test object, as suggested by Riesser,[2] and Chang and Gaddum.[3]

METHODS

The rectus abdominis of the frog was excised and suspended in a muscle chamber containing 20 c.c. of Ringer's solution (114 mM NaCl, 2 mM KCl, and 1.8 mM $CaCl_2$). After one hour the Ringer's solution was replaced for thirty seconds by a test solution containing substances causing contraction of the muscle. The height of contraction was registered by an isotonic lever on a kymograph. The muscle was then washed for ten minutes in Ringer's solution and the procedure repeated until three successive exposures to a test solution gave similar responses. At this point the counterbalance on the lever was adjusted so that the height of the reading on the kymograph was the same in the various experiments. Then the muscle was exposed to a series of test solutions for thirty seconds and washed with Ringer's solution for ten minutes.

In order to eserinize the muscle it was immersed for thirty minutes in a 0.0004 per cent physostigmine salicylate solution. Between exposures to the

*From the Department of Pharmacology, Cornell University Medical College, New York.
Received for publication, December 21, 1942.

various test solutions the eserinized muscle was washed first with Ringer's solution for five minutes and then with physostigmine for five minutes.

The test solutions used were:

(1) Acetylcholine solution containing 0.1 to 0.01 mg. per 100 c.c. with uneserinized muscles and 0.01 to 0.001 mg. per 100 c.c. with eserinized muscles.
(2) 20 to 60 mM potassium.
(3) Both acetylcholine and potassium.

Calcium was present in the various solutions in concentrations of from 0 to 180 mM. The solutions were rendered isotonic with Ringer's solution by the addition of NaCl. The pH was corrected to 7 when necessary by the addition of acid or basic phosphate. Air was bubbled through the solutions in the muscle chamber during the experiments.

The results were tabulated in millimeters of excursion of the lever as measured from the records.

TABLE I

ACETYLCHOLINE AND POTASSIUM CONTRACTIONS OF THE RECTUS ABDOMINIS MUSCLE IN RELATION TO THE CALCIUM CONCENTRATION

SUBSTANCE	NO. OF EXPTS.	AVERAGE CONTRACTION IN PER CENT OF THAT OBTAINED IN RINGER'S SOLUTION (Ca 1.8 mM) FOLLOWED BY STANDARD ERROR OF MEAN				
		18 mM $CaCl_2$	36 mM $CaCl_2$	54 mM $CaCl_2$	90 mM $CaCl_2$	180 mM $CaCl_2$
Acetylcholine*	25	90 ± 1.0	85 ± 0.9		80 ± 1.1	
20 mM Potassium	25	35 ± 0.9	16 ± 0.5		11 ± 1.1	
40 mM Potassium	25	55 ± 0.9	35 ± 0.9		11 ± 1.0	4 ± 0.7
60 mM Potassium	10			36 ± 1.1	18 ± 1.0	5 ± 1.0
Acetylcholine and 20 mM Potassium	10	65 ± 1.0			56 ± 1.0	
Acetylcholine and 40 mM Potassium	10		65 ± 1.0		55 ± 1.0	

*For concentration of acetylcholine see text.

RESULTS

The Effect of Acetylcholine in the Presence of Various Concentrations of Calcium.—The results, summarized in Table I and Fig. 1, show that an increase of the calcium content of the acetylcholine solution from 1.8 to 18 mM decreased the height of contraction caused by acetylcholine by about 10 per cent; and an increase of the calcium content to 90 mM decreased the height of contraction by about 20 per cent. Thus, varying the concentrations of calcium in the test solution had but little effect on the height of contraction caused by acetylcholine. This was true with both eserinized and uneserinized muscle, the only difference being in the absolute concentration of acetylcholine necessary to produce the contraction.

The Effect of Potassium in the Presence of Various Concentrations of Calcium.—The results are given in Table I and Fig. 1. They are expressed as a percentage of the response to solutions containing respectively 20, 40, and 60 mM potassium in the otherwise standard Ringer's solution (Ca, 1.8 mM). The

effectiveness of all these potassium solutions fell off rapidly as the calcium concentration was increased. At the higher potassium concentrations slightly more calcium was required to produce a given reduction in the response, but for all three concentrations 180 mM calcium caused a reduction in the average response to 5 per cent or less of the control value.

The Effect of Acetylcholine Plus Potassium in the Presence of Various Concentrations of Calcium.—The contracting effect of a solution containing acetylcholine and potassium is greater than one containing acetylcholine or potassium alone.

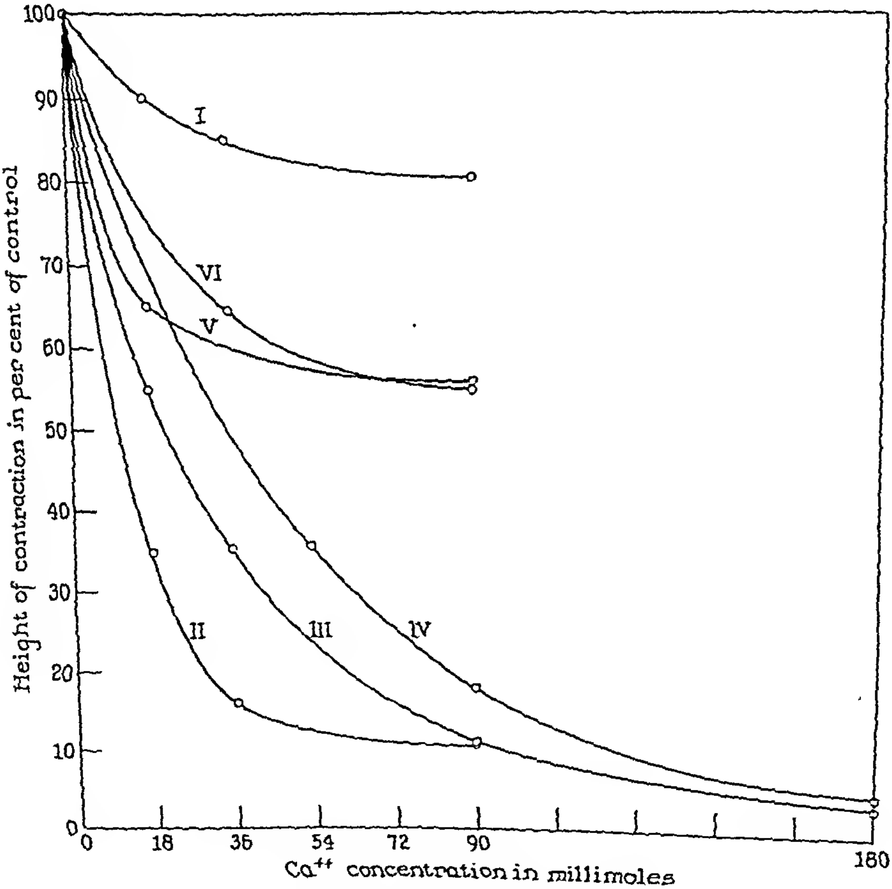


Fig. 1.—Influence of calcium on the contraction of the rectus abdominis muscle caused by acetylcholine and potassium. Values are expressed in percentage of the control response with muscle in Ringer's solution (1.8 mM Ca). The height of contraction in per cent is plotted against the calcium concentration in millimols. I, Acetylcholine (for concentration see text). *II*, 20 mM Potassium. *III*, 40 mM Potassium. *IV*, 60 mM Potassium. V, Acetylcholine and 20 mM Potassium. *VI*, Acetylcholine and 40 mM Potassium.

In the first series of experiments the muscle response to a solution containing 0.1 to 0.01 mg. acetylcholine per 100 c.c., 20 mM potassium, and 1.8 mM calcium was recorded. An increase in the calcium content to 18 mM decreased the height of contraction by about 35 per cent, and an increase of the calcium concentration to 90 mM caused a 44 per cent decrease. In the second series of experiments the muscle was exposed to a solution containing acetylcholine (0.1 to 0.01 mg. per 100 c.c.), 40 mM potassium and 1.8 mM calcium. An increase of the calcium con-

centration to 36 mM decreased the height of contraction by 35 per cent, and an increase of the calcium concentration to 90 mM caused a 44 per cent decrease. This effect was observed with both eserinized and uneserinized muscle, the only difference being the concentration of acetylcholine required to produce the contraction.

A series of experiments paralleling the experiments described above in which proteins were added to the test solution have been carried out with results similar to those summarized in Table I and Fig. 1.

The technique described may be used for an approximate determination of the acetylcholine and potassium content of an unknown solution by the following procedure: First, the eserinized rectus abdominis of the frog is immersed in the unknown solution, the height of contraction registered. Then to an aliquot, 90 mM calcium is added and the contracting effect of this solution is measured. If the increase in the concentration of calcium does not modify significantly the height of contraction, it may be assumed that the original solution contained acetylcholine and little or no potassium. If on the other hand the height of contraction is decreased by 80 or 90 per cent, the solution may be assumed to contain potassium but no acetylcholine. If an intermediate result is obtained, it may be assumed that both potassium and acetylcholine are present in the unknown solution.

The method can be used for quantitative estimation of the acetylcholine or potassium content of an unknown solution:

If the solution contains acetylcholine alone, the acetylcholine content can be estimated by comparing the height of contraction of the muscle immersed in the unknown solution with the height of contractions caused by known solutions.

If the solution contains potassium without acetylcholine, a determination of the amount of potassium is possible by varying the calcium concentration of the solution. If the addition of 36 mM calcium decreased the height of contraction by about 80 per cent, it may be assumed that the unknown solution contains about 20 mM potassium. If the decrease is 65 per cent, the original solution probably contains 40 mM potassium, and similarly if the decrease is 50 per cent, the solution probably contains 60 mM potassium.

With a solution that contains both acetylcholine and potassium a similar procedure can be carried out. For example, if the addition of 18 mM calcium to the unknown solution results in a 35 per cent decrease in the height of contraction, it may be assumed that the solution contained about 20 mM potassium. Similarly if the addition of 36 mM calcium to the unknown solution results in a 35 per cent decrease of the height of contraction, the solution contained about 40 mM potassium. After estimation of the concentration of potassium of the unknown fluid, the acetylcholine content can be estimated by the usual method indicated above. The height of contraction caused by the unknown solution must be compared with the height of contraction produced by known solutions of acetylcholine dissolved in the same concentration of potassium as the unknown solution.

The potassium content of the unknown solution may also be determined by plotting the height of contraction of the rectus abdominis, as expressed in percentage of the first contraction, against the increase of the calcium content of

the fluid. By a gradual increase of the calcium content from zero to a high concentration, the curve obtained is a hyperbola. As the ratio of the K/Ca content of the solution decreases towards unity, the curve falls sharply. As the ratio becomes less than unity, the curve starts to level out and approximate asymptotically the base line.

The body fluids contain buffers, most of which form insoluble salts with calcium; therefore, a correction must be made for the amount of calcium precipitated. This can be accomplished by adding calcium in small amounts until no further precipitate is formed. Any further amounts of calcium added remain for the most part in solution and are available to counteract the effect of potassium. The pH of all solutions must be corrected to the same level, since the height of contraction varies with the pH.

Immersion of the muscle for a few minutes in Ringer's solution is sufficient to reverse the effect of a few minutes' immersion in calcium.

The method was tested for urine. Acetylcholine (0.1 to 0.001 mg. per 100 c.c.), potassium (20 to 50 mM) or both acetylcholine and potassium were dissolved in urine and the added amount determined. The difference between the amount of substances added and that estimated by the muscle test was less than ±15 per cent.

DISCUSSION

This paper offers a method of determining the approximate content of acetylcholine and/or potassium in an unknown solution based on the effect of calcium in counteracting the effect of potassium on the rectus abdominis muscle.

The method is only approximate because of the following sources of error:

(1) The calcium-potassium antagonism on muscle contraction is not absolute.
(2) The original calcium ion content of the unknown solution is not known.
(3) It is difficult to estimate the exact amount of ionized calcium in case some of the added calcium precipitates.
(4) The unknown fluid might contain other substances which cause muscle contraction and which are more or less influenced by calcium.

The above studies prove that the effect of acetylcholine as a contraction-producing agent is somewhat modified by an increase in the concentration of calcium in the surrounding fluid. They also prove that the contraction of the muscle is due not to an excess of potassium per se, but to an increase of the K/Ca ratio. The calcium-potassium antagonism is not a simple relationship, since exceedingly large amounts of calcium are necessary to counteract completely the effect of potassium.

SUMMARY

(1) A biologic method for the rapid estimation of acetylcholine and/or potassium content of body fluids is offered. The method is based on the antagonism between calcium and potassium on the contraction of the rectus abdominis muscle of the frog.

(2) The contracting effect of acetylcholine on the muscle is but slightly influenced by a large excess of calcium.

(3) The contraction caused by potassium can be prevented almost completely by a large amount of calcium.

REFERENCES

1. Lánczos, A.: Trennung von Azetylcholin und Kalium in Flüssigkeiten und Gewebsauszügen, Arch. f. exper. Path. u. Pharmakol. 196: 322, 1940.
2. Riesser, O.: Über die durch Azetylcholin bewirkte Erregungskontraktur des Froschmuskels und ihre antagonistische Beeinflussung durch Atropin, Novokain und Kurare, Arch. f. exper. Path. u. Pharmakol. 91: 342, 1921.
3. Chang, H. C., and Gaddum, J. H.: Choline Esters in Tissue Extracts, J. Physiol. 79: 255, 1933.

A STUDY OF PRESERVED COMPLEMENT*

PAUL FUGAZZOTTO, M.S., GRAND RAPIDS, MICH.

THE most uncertain factor in complement fixation tests has always been the complement itself, since it is practically impossible to predict how long any particular lot of freshly drawn guinea pig serum will retain its potency. Especially distressing is the instability of complement diluted for tests; and it is perhaps because of this instability that some attempts at complement fixation have met with little success.

During recent years, however, several workers have attempted to reduce the factor of uncertainty by various methods of preservation. Ginsburg and Kalinin[1] reported satisfactory preservation of guinea pig complement for three to four months by addition of 100 mg. sodium chloride and 40 mg. boric acid per c.c. of serum. The method used in the present study is based on the one described by these authors and differs mainly in that 80 mg. instead of 100 mg. of sodium chloride were used.

PREPARATION OF PRESERVED COMPLEMENT

For each pool of complement serum, 8 to 10 c.c. of blood were drawn by cardiac puncture from each of four or more healthy adult guinea pigs, under ether anesthesia. The blood was collected separately in sterile Petri plates, left at room temperature for an hour, and in the cold room four to twenty hours. (Petri plates were used to allow greater surface for evaporation of the ether with which the blood becomes saturated during anesthesia.) The clots were carefully removed, avoiding hemolysis. The clear sera were obtained by the minimum amount of centrifugation and placed immediately in the cold room. Within twenty-four hours after bleeding the animals, a portion of each serum was removed for titration according to the technique given later. In this titration, unless there was complete hemolysis of one unit of sensitized sheep cells in the presence of less than 0.05 c.c. of the 1:15 diluted complement, the latter was not included in the pool.

The satisfactory sera were pooled in a large sterile test tube fitted with a cotton plug. After the volume had been determined, 80 mg. of sodium chloride

*From the Michigan Department of Health Laboratories, Western Michigan Division.
Received for publication, January 28, 1943.

and an excess (40 mg.) of pulverized boric acid were added per c.c. of serum. If it were not possible to titrate the sera within twenty-four hours, proportionate amounts of the salts were added to the individual sera; the mixtures were shaken till it was certain that all the sodium chloride had dissolved; and the titrations were done later. Since boric acid dissolves very slowly, the mixture was agitated about three times daily for two days to insure maximum solubility. When the odor of ether was no longer detectable in the serum, the cotton plug was replaced by a cork stopper to prevent further evaporation.

PREPARATION OF THE REAGENTS FOR COMPLEMENT TITRATIONS

Washed Sheep Blood Cells.—The cells of freshly drawn sheep blood were washed four times in large volumes of physiologic salt solution, and a 5 per cent suspension was made in saline.

Sensitized Sheep Cells.—The sheep cells were sensitized by mixing the required volume quickly and thoroughly with an equal volume of amboceptor diluted according to titer. The suspension was again agitated every five minutes for fifteen minutes, at which time it was considered ready for use and was discarded after one and one-half hours. Of this suspension 0.2 c.c. were used as one unit.

Titration of Amboceptor.—The amboceptor titer was determined as the highest dilution of amboceptor to the nearest 100, producing complete hemolysis of the cells in the presence of 0.05 c.c. of 1:15 diluted complement.

Because any slight error in the preparation of dilutions might result in amboceptor of varying potency and because the blood cells of different sheep vary somewhat in fragility, care was taken in so far as possible to standardize these two reagents, thereby leaving the complement as the only significant variable thoroughout the period of the experiments.

Complement.—Throughout the entire period of this work the complement sera were used at a dilution of 1:15. Dilutions of unpreserved complement were made with physiologic saline. Preserved complement was first made isotonic to normal serum by diluting it 1:10 with distilled water, and then made up to 1:15 with physiologic saline.

TECHNIQUE OF COMPLEMENT TITRATIONS

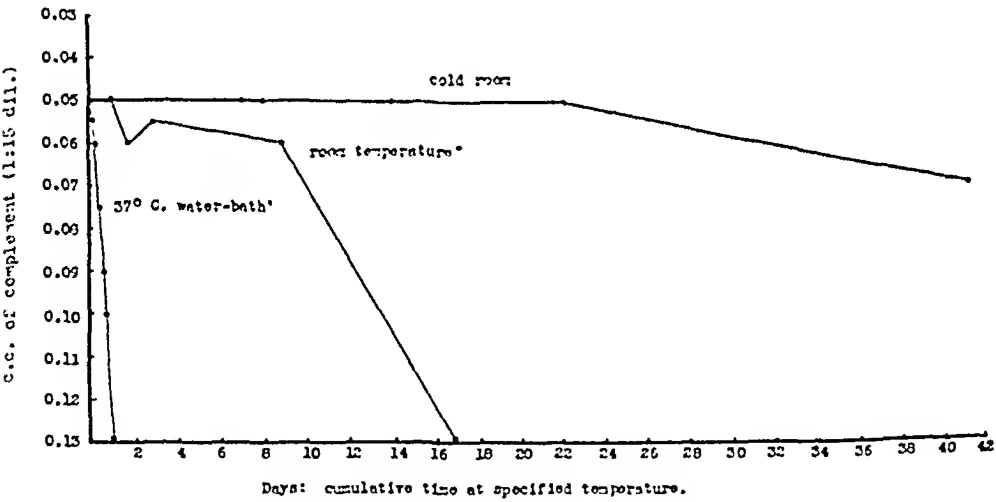
A series of several tubes was set up for each specimen of complement, and various amounts of the diluted complement were pipetted to the bottom of the tubes. To each tube was added 1 unit of sensitized sheep cell suspension. As each rack of tests was completed, it was shaken thoroughly, and placed immediately in a water bath at 37° C. for fifteen minutes. The titer recorded was the smallest volume of diluted complement, to the nearest 0.005 c.c., necessary for complete hemolysis of the sensitized sheep cells, as indicated by simple inspection.

EFFECT OF AGE AND TEMPERATURE ON THE STABILITY OF PRESERVED COMPLEMENT

At varying intervals over a period of sixteen months, complement titrations were made on 29 pooled guinea pig sera of varying ages, accumulated during this time and stored continuously in the cold room at 5° to 8° C.

One of these sera, already two and one-half months in storage, was selected to determine the effect of intermittent exposure to higher temperatures. This complement was divided into three portions, the first of which was kept continuously in the cold room. The second and third portions were also stored in the cold room when not in use; but at some arbitrarily set time before titration, the second portion was transferred to room temperature (25° to 32° C.), and the third to a 37° C. water bath. Then samples of each portion were diluted 1:15 for titrations.

Results.—In considering analysis of Graph I, it must be remembered that the complement used was already two and one-half months old at the first point plotted. The "cold room" curve, which followed a course similar to those obtained for all the pooled sera tested, continued a straight line for twenty-two days more. No test was made between the twenty-second and forty-first days, but tests with other sera indicated that the curve would have continued a straight line to the thirtieth or thirty-fifth day. Hence, preserved complement kept in the cold room would retain its activity practically constant for three to four months.



Graph 1.—Effect of temperature on stability of undiluted preserved complement indicated by c.c. of 1:15 dilution required for complete hemolysis of 1 unit of sensitized cells.

The complement was already 2.5 months old at the beginning of the experiment.

*Sera were not left continuously at the temperature specified, but stored in the cold room between test periods.

On the other hand, intermittent exposure of the complement to higher temperatures increased the rate of deterioration, as indicated by the more rapid drop in the other two curves.

EFFECT OF AGE AND TEMPERATURE ON STABILITY OF DILUTED PRESERVED COMPLEMENT

Samples of several preserved sera were diluted 1:15. The dilutions were stored in the cold room and titrated at intervals of approximately twenty-four hours for several days.

One of these diluted sera, selected to determine the effect of intermittent exposure to higher temperatures, was divided into three portions. The first of these was kept continuously in the cold room; the second and third were also

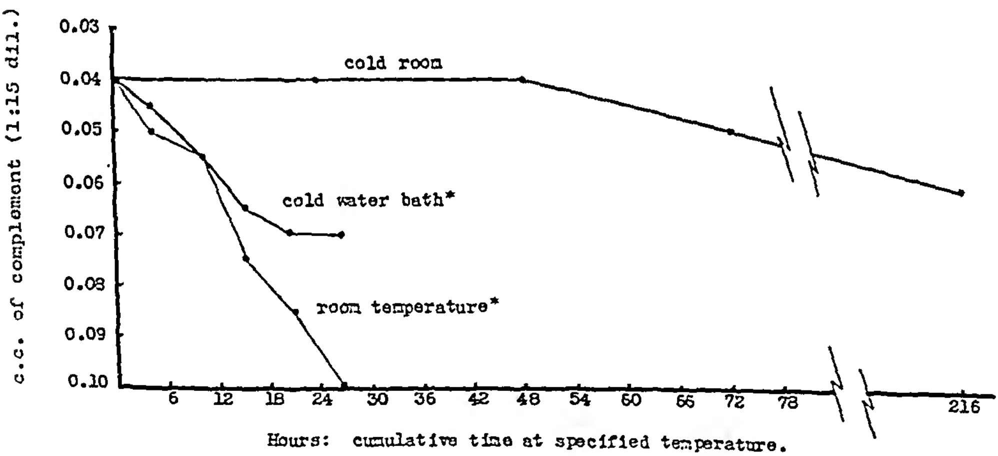
stored in the cold room when not in use. At some arbitrarily set time before titration, the second portion was transferred to a cold water bath (10° to 20° C.) and the third to room temperature (25° to 32° C.). Then samples of each were taken for titration.

Results.—The "cold room" curve in Graph II indicates that the dilution of preserved complement kept continuously in the cold room retained practically constant potency for twenty-four to forty-eight hours. This was also true of the several other diluted sera tested, some of which retained their potency still longer.

On the other hand, intermittent exposure of diluted complement to higher temperatures increased the rate of deterioration as indicated by the more rapid drop in the other two curves.

DISCUSSION

It has long been known that freshly drawn guinea pig complement can no longer be used three or four days after drawing. Furthermore, it has been the experience in the present study that a dilution of unpreserved complement demonstrated appreciable deterioration within eighteen to twenty hours even if kept at cold room temperature.



Graph II.—Effect of temperature on stability of diluted (1:15) preserved complement, indicated by number of c.c. required for complete hemolysis of 1 unit of sensitized cells.

*Sera were not left continuously at the temperature specified, but stored in the cold room between test periods.

From the data presented it seems evident that complement prepared according to the method described will remain satisfactory for a much longer period of time (three to four months) comparing favorably with the complement of Ginsburg and Kalinin. For best results, however, it is necessary to avoid exposure of the complement to temperatures above 5° to 8° C. for any considerable period of time, especially during the first twenty-four hours after obtaining the blood from the guinea pigs; that is, during the time when the salts have not yet been added. The stability of complement seemed to depend in great degree upon its plane of potency at the time the preservatives were added, an occasional pool retaining satisfactory activity for as long as six months. From this it also followed that the stability of the diluted complement depended upon the status of the pool from which it was made. A dilu-

tion of a very poor pool, one that had aged considerably or one that was poor at the very beginning, was likely to be less stable than a dilution of a good pool.

Complement preserved by freezing and drying, according to Flosdorf, Boerner, Lukens, and Ambler,[2] apparently retains its activity for periods as long as three years. The later method of Boerner, Flosdorf, and Lukens,[3] of freezing and drying after addition of sodium acetate and boric acid, also has its advantages. But the advantage of a method like the one described in this study is that it does not necessitate having special apparatus which might be too cumbersome as well as too expensive for a small laboratory. Furthermore, it is rather important to know how the frozen complement behaves after dilution for use. That is, if a dilution of this complement is only as stable as a dilution of fresh complement, it would seem from the results of the present study that there is an added advantage in the salting method.

SUMMARY

Data are presented concerning the nature of complement preserved with sodium chloride and boric acid.

Complement preserved by the method described retains its activity for three to four months. An occasional pool was found satisfactory for as long as six months.

This complement diluted to titer retained its activity almost undiminished for twenty-four to forty-eight hours when kept at 5° to 8° C.

The importance of temperature is stressed, and a cold water bath is suggested for complement (diluted or undiluted) that must be kept out of the cold room for any length of time.

CONCLUSION

The use of sodium chloride and boric acid in the manner described is a satisfactory method of preserving guinea pig complement. The use of complement so preserved should tend to increase the uniformity and dependability of the results of complement fixation tests.

REFERENCES

1. Ginsburg, S., and Kalinin, W.: Zur Frage der Komplementkonservierung, Ztschr. f. Immunitätsforsch. u. exper. Therap. 63: 107, 1929.
2. Flosdorf, W. E., Boerner, F., Lukens, M., and Ambler, T. S.: Cryochem-Preserved Complement of Guinea Pig Serum, Am. J. Clin. Path. 10: 339, 1940.
3. Boerner, F., Flosdorf, E. W., and Lukens, M.: Stabilization of High Vacuum-Dried Guinea Pig Complement Before Drying and After Restoring With Water, Am. J. Clin. Path. 11: 122, 1941.

AN IMPROVED FEEDER FOR GUINEA PIGS*

STEWART H. WEBSTER, PH.D., ERVIN J. LILJEGREN, AND DAVID J. ZIMMER
BETHESDA, MD.

THE frequent necessity for accurately measuring the quantity of pellet food consumed by guinea pigs on experiments led to the development of a type of feeder which has been in use in this laboratory for nearly a year. This device offers several advantages over the old style of open box. (1) Contamination of food is reduced to a minimum since the feed opening is adjustable to the size of the head. (2) Very little food is lost since the animal has to insert its head

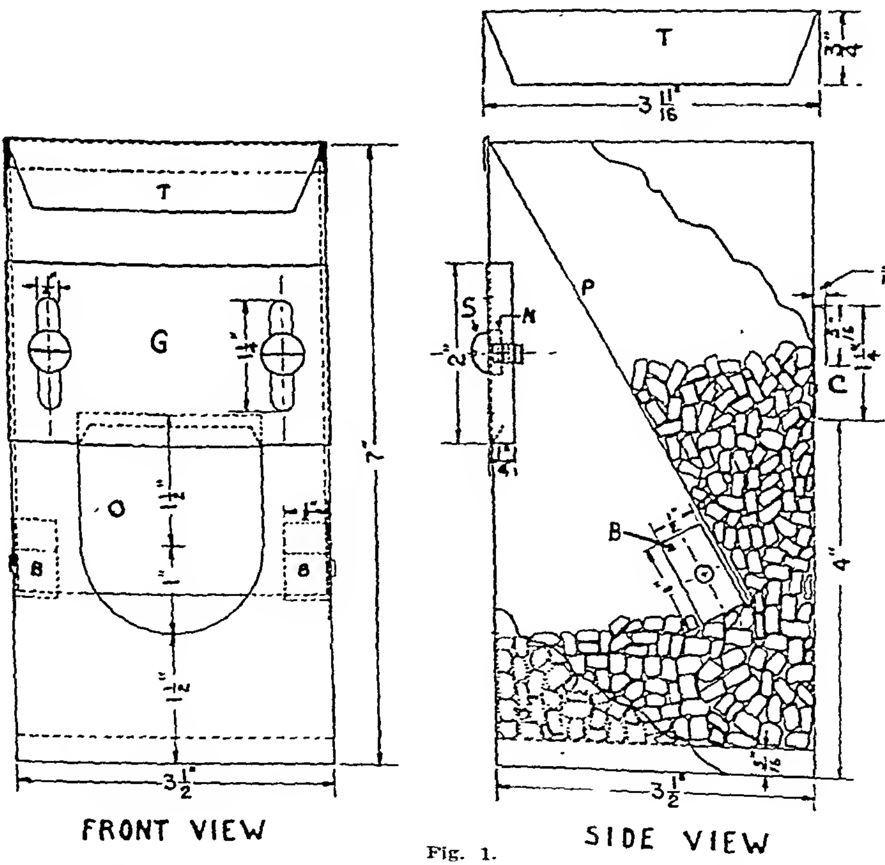


Fig. 1.

into the opening in order to secure food. (3) The capacity of the feeder, about 550 Gm. of pellet food, is sufficient for four or five animals for several days. (4) By reducing contamination and food loss the use of these feeders results in a considerable decrease of food cost. This saving should compensate for the difference in cost between these feeders and open cups.

*From the Division of Industrial Hygiene, National Institute of Health, United States Public Health Service, Federal Security Agency.

Received for publication, January 15, 1943.

Although feeders made of wood or masonite are serviceable for a limited time, ones constructed of sheet iron are resistant to chewing by the animals. The accompanying drawing shows details and dimensions for feeders made of No. 22 gauge galvanized iron. The box, consisting of four sides with a bottom soldered in, has a smoothly formed feed opening, *O*, the size of which is regulated by the slotted gate, *G*, which is adjustable and can be locked in position by the screws, *S*, and nuts, *N*, soldered to the side. The bottom of the gate is bent upward and backward in order to eliminate any sharp edge which might cut the animal. The plate, P, 5¾ inches long by 3⅜ inches wide, keeps the feed in the hopper and is removable for cleaning. The top of this plate is bent as shown, and the lower part is held in position by the brackets, *B*, soldered and riveted to the sides. The position of these brackets governs the throat size at the bottom of the plate and consequently the flow of pellets. About ¾ inch is satisfactory for this throat width using cylindrical pellets having diameters of about $^3/_{16}$ inch and lengths ranging from ⅛ to ¼ inch. The feeder is covered with a top, *T*, the edges of which are bent inward so that friction prevents the animal from knocking it off in case it should climb on top. Finally, the clip, *C*, riveted to the back, serves to hold the feeder securely to the cage and prevents upsetting.

We wish to thank Mr. R. R. Groomes, Foreman of the Sheet Metal Shop, for suggestions in the design of this feeding device.

A RAPID METHOD FOR DETERMINING THE SULFONAMIDE DRUG OF CHOICE*

ROBERT J. ATWELL, A.B., RICHARD P. SEXTON, A.B., AND MARY A. POSTON, M.A.
DURHAM, N. C.

IN SULFONAMIDE therapy, the choice of the drug, its dosage, its effective blood level, its reactions, etc., depend both on the patient and the infecting organism. Bactericidal tests of the patient's organism with the various sulfa drugs are desirable so that the most effective one can be selected as quickly as possible. A preliminary report describing a rapid method for the determination of the drug of choice in cultures of the blood, spinal fluid, nose and throat, sputum, and wounds is presented.

MATERIALS AND METHODS

Sulfanilamide, sulfapyridine, sodium sulfapyridine,† sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine were chosen for the experiments because of their adaptability to clinical use. Stock solutions of the drugs were made in beef-infused buffered broth,‡ pH 7.4,[1] using a 100 mg. per cent concentration of sulfanilamide and the sodium salts, and a 15 mg. per 100 c.c. concentration of sulfapyridine, sulfathiazole, and sulfadiazine.

*From the Department of Bacteriology, Duke University School of Medicine and Duke Hospital.

Received for publication, February 3, 1943.

†The sodium salts were used so that higher concentration of the drugs could be obtained. Since the same concentration of the drug and that of its sodium salt are not always comparable in their inhibition effects, both forms of the drug were used for these experiments. At the present time, no explanation can be given of this discrepancy, which has been noted in previous experiments.

‡See footnote on page 1621.

Buffered beef-infused (3.5 per cent) agar,* pH 7.4 was used as the stock medium for pouring plates. This was tubed in 8 c.c. amounts, to which were added varying amounts of the broth filtrates of the drugs. The final concentrations of the drugs used in the experiments were 20, 10, and 5 mg. per cent of the sodium salts; 20 and 10 mg. per cent of sulfanilamide, and 5 mg. per cent of sulfapyridine, sulfathiazole, and sulfadiazine.

BLOOD

Fifteen cubic centimeters of the patient's blood are introduced into a flask containing 4 c.c. of 2.5 per cent sodium citrate solution. One cubic centimeter of the citrated blood is added to each tube containing molten agar (cooled to 40° C.), sulfonamide solution, and broth. Control plates contain agar but no sulfonamide. If the patient is receiving chemotherapy, one plate is poured using agar containing 1 mg. per 100 c.c. para-amino-benzoic acid. The cultures are incubated at 37.5° C.

TABLE I

CHART SHOWING INHIBITION OF GROWTH OF PNEUMOCOCCUS, TYPE 27, IN CULTURE OF BLOOD AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10*	20	5	5	10	20	5	5	10	20	5	5	10	20
0	0	++	+++	++++	++++	+++	+++	++++	++++	0	0	0	0

*Milligrams per cent.
++++ = Complete inhibition.
+++ = 75 per cent inhibition.
++ = 50 per cent inhibition.
+ = 25 per cent inhibition.

TABLE II

CHART SHOWING INHIBITION OF GROWTH OF BETA STREPTOCOCCUS IN CULTURE OF NOSE AND THROAT AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
++++	++++	++	++	+++	+++	0	0	0	0	0	0	0	0

After eighteen hours' incubation, if colonies are visible in the control plates, colony counts are made of the controls and the plates containing the drugs. Smears of the colonies are made and stained by Gram's method and the organism is identified. By this method, the drug of choice can be determined at the time the cultures are positive. (See Table I.)

NOSE AND THROAT

The swabs are placed immediately into tubes containing 10 c.c. of beef-infused broth. As much material as possible is washed from the swabs by rolling them against the inside wall of the test tube. One tenth cubic centimeter of the broth is transferred to another tube containing 10 c.c. of broth and mixed thoroughly (Tube 2). The sulfonamide, 1.5 c.c. sterile sheep's blood,

*Broth and agar containing 1 per cent bacto-peptone (Difco brand) were used instead of peptone-free media, as Gay and his co-workers[2] have shown that sulfanilamide is bacteriostatic in the presence of bacto-peptone. When media containing less than 1 per cent bacto-peptone is used, forty-eight to seventy-two hours' incubation of the cultures is required *before* colonies appear in the plates.

0.1 c.c. of the broth mixture from Tube 2, and sufficient stock broth to bring the volume up to 15 c.c. are added to each tube of molten agar (cooled to 40° C.), and after thorough mixing, plates are poured. Control plates are poured using agar without drug.

After eighteen hours' incubation, the plates are examined for organisms. If present, colony counts are made and the organisms are identified. (See Table II.)

SPINAL FLUID

The spinal fluid is centrifuged at high speed for fifteen minutes. After centrifugalization, the supernatant fluid is discarded, and a smear is made of the sediment and stained by Gram's method. A tube containing 10 c.c. of blood broth is inoculated with two to four loopfuls of the sediment, depending upon the number of organisms present in the smear, and is incubated at 37.5° C. for thirty minutes. The sulfonamide solution, 1.5 c.c. of sterile sheep's blood, 0.1 c.c. of the broth culture of the spinal fluid, and enough stock broth to bring the volume up to 15 c.c. are added to each tube of molten agar (cooled to 40° C.), and after thorough mixing, plates are poured. Control plates are poured using agar without drug. The cultures are incubated at 37.5° C.

TABLE III

CHART SHOWING INHIBITION OF GROWTH OF PNEUMOCOCCUS, TYPE 17, IN CULTURE OF SPINAL FLUID AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
+	+	++++	++++	++++	++++	++++	++++	++++	++++	+-	++	++++	++++

TABLE IV

CHART SHOWING INHIBITION OF GROWTH OF STREPTOCOCCUS HEMOLYTICUS IN CULTURE OF SPUTUM AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFANILAMIDE		SULFAPYRIDINE	SODIUM SULFAPYRIDINE			SULFATHIAZOLE	SODIUM SULFATHIAZOLE			SULFADIAZINE	SODIUM SULFADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
++++	++++	++	++++	++++	++++	++++	0	++	++++	++	++	++	++

After eighteen hours' incubation, the plates are examined, the in vitro effectiveness of the sulfonamides is determined, and the organisms are identified. (See Table III.)

SPUTUM

A smear is made of the sputum and stained by Gram's method. One or two loopfuls of the sputum, depending upon the number of organisms present in the smear, are inoculated into a tube containing 10 c.c. of broth. One-tenth cubic centimeter of a 1:100 dilution of the sputum broth mixture is used as the inoculum, and plates are poured, as described above.

After eighteen hours' incubation, the plates are examined for organisms. If colonies are present, the bacteriostatic effect of the sulfonamides is determined and the organisms are identified. (See Table IV.)

WOUNDS

The swabs are placed immediately into a tube containing 10 c.c. of broth. As much material as possible is washed from the swabs by rolling them against

the inside of the test tube. A smear is made of the broth and stained by Gram's method. Dilutions are made according to the number of organisms in the stained smear. One-tenth cubic centimeter of the final dilution of broth is used as the inoculum, and plates are poured, as described above.

After eighteen hours' incubation, the plates are examined, the in vitro effectiveness of the sulfonamides is determined, and the organisms are identified. (See Table V.)

DISCUSSION

The drug of choice can be determined at the time (eighteen to twenty-four hours) the cultures are positive for growth. In that length of time the organisms most likely have not become drug-fast. Various concentrations of the drugs are used, so that the approximate blood level, necessary for therapeutic effectiveness can be estimated at the same time. Identification of the organisms can be made from the control plates of the experiment, so that duplicate cultures are not necessary.

If the patient's blood contained a high concentration of one of the sulfonamides at the time of the experiments, none of the plates may be positive for growth, since the medium does not contain para-amino-benzoic acid, but growth will appear in the plate to which 1 mg. per 100 c.c. para-amino-benzoic acid has been added. This was not encountered in any of our experiments.

TABLE V

CHART SHOWING INHIBITION OF GROWTH OF HEMOLYTIC STAPHYLOCOCCUS AUREUS IN CULTURE OF WOUND AFTER EIGHTEEN HOURS' EXPOSURE TO SULFONAMIDES

SULFA-NILAMIDE		SULFA-PYRI-DINE	SODIUM SUL-FAPYRIDINE			SULFA-THIAZOLE	SODIUM SULFATHIAZOLE			SULFA-DIA-ZINE	SODIUM SUL-FADIAZINE		
10	20	5	5	10	20	5	5	10	20	5	5	10	20
0	0	0	0	0	0	++++	0	++++	++++	0	0	0	0

The experiments included cultures of blood, nose, and throat, spinal fluid, sputum, and wounds. From these cultures the following bacterial types and strains were isolated: *Diplococcus pneumoniae, Streptococcus hemolyticus,* and hemolytic *Staphylococcus aureus.* This method proved equally efficient for all organisms.

An attempt to correlate the in vitro experiments with the in vivo effectiveness of the sulfonamides is in progress.

CONCLUSIONS

1. A simple, quick method for testing the inhibiting effects in vitro, of sulfanilamide, sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine upon the growth of various bacterial types and strains is described.

2. The drug of choice can be determined as soon as the control cultures are positive.

REFERENCES

1. Poston, M. A., and Orgain, E. S.: A Comparison of the Bacteriostatic Effect of the Sulfonamide Drugs Upon the Growth of 25 Strains of Streptococcus Viridans, Am. J. M. Sc. 203: 577, 1942.
2. Gay, F., Clark, R., Street, A., and Miles, W.: The Mode of Action of Sulfanilamide on Streptococcus. II., J. Exper. Med. 69: 607, 1939.

A SIMPLIFIED METHOD FOR FEEDING RATS

H. Ward Ferrill, Ph.D., Chapel Hill, N. C.

A SATISFACTORY method for placing known quantities of solutions directly into the stomach of white rates becomes more and more important when the number of animals is large and laboratory assistants are unavailable. The author has found the following method very satisfactory over a period of several years; it is simple and eliminates practically all danger of injury to the animal.

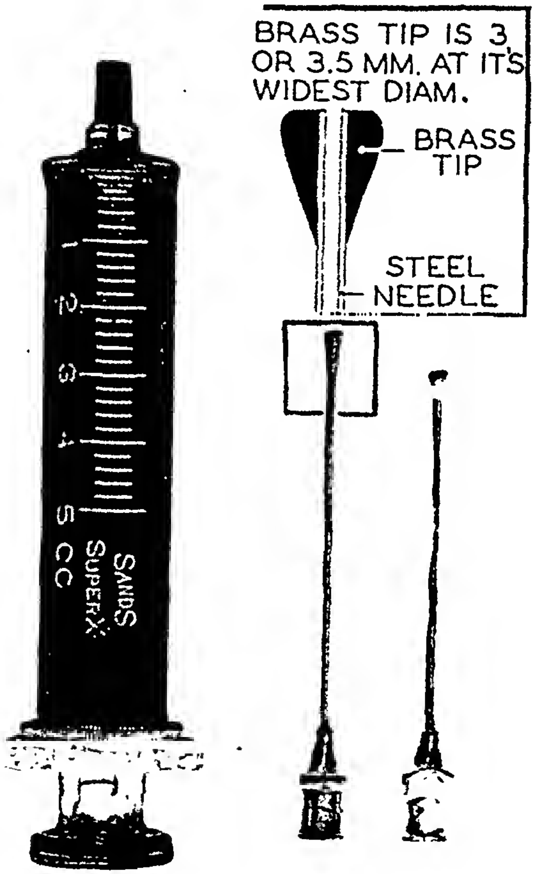


Fig. 1.

An ordinary hypodermic syringe of any desired capacity is used for the accurate measurement of the material. A long, large or small bore needle is used and on its end a small brass knob is soldered. The brass extends far enough up the needle to give it a good area of contact, and an opening the same

*From the Department of Physiology, University of North Carolina School of Medicine. Received for publication, January 29, 1943.

size as the bore of the needle is made through the center of the attached metal. The brass is polished to a very smooth finish so that it can be inserted easily and irritation of the esophagus or cardiac sphincter avoided. It has been found helpful to have different sized needles and knobs for various sized rats if the work is begun when the animals are small. The size which will slip easily down the esophagus of half-grown or adult rats is too large for young animals. The diameters of the knobs used by the author range from 3.0 to 3.5 mm., the smaller being satisfactory for rats of weaning age. A needle at least 2.5 inches in length is more satisfactory than shorter ones.

The animal is held in the left hand with the fingers well forward on the neck to tilt the head back slightly. Then the needle is passed directly down the esophagus into the stomach, and the solution is expelled by pushing in the plunger of the syringe. The animal may clamp the needle with its teeth, but this does not interfere as it does when using a rubber catheter. No gag is necessary for inserting the needle.

Many thousands of injections have been made with this procedure without a single case in which the needle passed into the trachea. The method is very rapid; one person can feed as many as 120 animals per hour after very little training. Autopsies on experimental animals indicate that there is no gross or microscopic change in the esophageal mucosa from daily use of this procedure.

Fig. 1 shows the relative proportions of the various parts of the instrument.

A RAPID METHOD OF PREPARING PNEUMOCOCCAL SPECIFIC CARBOHYDRATES WITH THE AID OF AN ELECTRIC CURRENT*

MARTIN BURGER, M.SC., NEW YORK, N. Y.

THE methods generally used to prepare soluble specific substance of pneumococci[1-3] require autolysis of the cocci. From the autolyzate containing the specific soluble substance and large amounts of protein, the latter is removed. Both the autolysis and the elimination of protein are time-consuming, and we thought it worth-while to investigate a method which would require neither autolysis nor the removal of large amounts of protein.

In 1935, Yen and Kurotchkin[4] and Murao and Morimoto[5] showed that immunologically specific substances could be prepared by electrolyzing suspensions of certain organisms. The former authors obtained the soluble specific polysaccharides of Friedländer's bacillus and *Monilia tropicalis;* and the latter authors obtained a clear filtrate from a suspension of *Bacillus typhosus,* which was said to be more efficient for immunization of mice than a corresponding heat-killed suspension of the same organism.

EXPERIMENTAL

Recent mouse-passed cultures of Types II, III, VI, XII, XIII, XIV, XIX, and XXIII pneumococci were grown for 18 hours in beef-heart broth medium containing 3 per cent of normal horse serum. The organisms were collected

*From the Bureau of Laboratories, Department of Health.
Received for publication, February 17, 1943

by centrifugation and washed twice with 0.85 per cent saline. The average yield of packed cells from ten liters of broth was 15 c.c. The cells were suspended in 150 c.c. of 0.85 per cent NaCl solution, transferred to a 600 c.c. beaker, and layered with 10 c.c. of toluol. A pair of platinum wire electrodes (B. & S. 19 gauge) were placed 6 cm. apart and immersed in the suspension. A variable resistance or bank of lamps in series with the electrodes served to control the voltage across the electrodes. The voltage was maintained between 18 and 22, and the current was 0.45 amperes. A water bath around the beaker was kept between 20° and 22° C., and a thermometer, to note the temperature and at the same time used as a stirrer, was kept in the suspension throughout the electrolysis. The temperature was maintained below 30° C. at all times. The solution was stirred every half-hour in order to remove bacterial protein substances which accumulated on the electrodes. The electrolysis was stopped when the solution became almost transparent. The time required for the dissolution of 15 c.c. of organisms was usually 20 to 24 hours. Most of the precipitated protein which formed was in the toluol layer, and the small amount of suspended matter proved, on microscopic examination, to be bacterial debris and some intact organisms. The latter, however, gave negative Neufeld quellung reactions.

The solution was separated from most of the precipitate by centrifugation, then filtered through filter paper, and the slightly turbid filtrate was further clarified by centrifugation in an angle centrifuge. The clear supernatant was dialyzed for 18 hours against running tap water and finally concentrated in vacuo to one-fifth the original volume of suspension. Any insoluble matter which formed was eliminated by filtration. A small portion of the clear filtrate, when tested for specific polysaccharide, gave an immediate heavy precipitate in homologous antiserum, but not in heterologous antisera. To the main filtrate were added 3 volumes of 95 per cent ethanol. A flocculent precipitate formed, which increased in amount on the addition of 5 c.c. of saturated sodium acetate solution containing 0.5 c.c. of glacial acetic acid. The precipitate, after settling in the refrigerator, was separated by centrifugation, dissolved in 25 c.c. of distilled water, and a 1 c.c. sample tested for the presence of protein. If protein was present (i.e., positive biuret or trichloracetic acid tests), the main solution was shaken with chloroform-butyl alcohol mixture, as recommended by Sevag.[6] Six extractions with the chloroform reagent usually removed all of the protein, as evidenced by negative biuret and trichloracetic acid tests. The polysaccharide was reprecipitated by adding 2 volumes of ethanol, allowed to stand in the refrigerator overnight, and centrifuged the following day. The alcoholic supernatant was discarded, and the precipitate suspended in 95 per cent ethanol, filtered on a Buchner funnel, washed with absolute alcohol, ether, and finally dried in a vacuum desiccator. The average yield of specific carbohydrate from the organisms grown in 10 liters of medium was 125 milligrams.

The substances were type-specific, as no cross reactions were obtained in heterologous antipneumococcal sera. They all gave a positive precipitin reaction in a dilution of 1:10[6] when tested against homologous rabbit antiserum. In the dry state, the substances were yellowish in color and readily soluble in water. One per cent solutions gave negative Millon, Hopkins-Cole, trichloracetic

acid, ninhydrin, biuret, and xanthoproteic acid tests for protein. The Molisch tests were positive, and the Fehling tests before acid hydrolysis were negative. After acid hydrolysis, the Fehling tests were positive.

A control suspension of organisms of each of the types studied was transferred to a thin-walled test tube without electrodes and kept immersed in the main suspension during the electrolysis. The nondissolution of the control organisms was confirmed by negative precipitin reactions on the supernatant after separation of the organisms by centrifugation.

The effect of different electrolytes on the dissolution of pneumococci with the aid of an electric current was also studied. One per cent solutions of $NaCl$, $NaNO_3$, Na_2SO_4, or $NaC_2H_3O_2$ were used as the suspending media. Four, 0.5 c.c. portions of packed Type III organisms (Type XIV and Type II organisms were treated similarly) were transferred to tubes, and each sample of cells was washed twice with one of the appropriate electrolytes. The washed cells were then suspended in 30 c.c. of the appropriate electrolyte, layered with 5 c.c. of toluol, subjected to the electric current, and treated as described above. With each electrolyte, dissolution of the organisms was complete at the end of four hours, as evidenced by the complete clearing of the suspension. Microscopic examination of the precipitate which formed showed only bacterial debris present. Chemical examination proved that the precipitate contained protein. One-tenth cubic centimeter portions of the clear aqueous solution gave positive precipitin reactions with homologous antiserum and no reactions with heterologous antisera. Control tubes containing 2 c.c. of the original suspension and not subjected to electrolysis remained milky.

After each suspension had cleared, it was filtered on a wet filter paper to retain the toluol and most of the precipitate. The cloudy filtrate was then passed through a Berkefeld N candle, and the clear filtrate on diluting with two volumes of 95 per cent alcohol gave a precipitate, which, after standing several hours in the refrigerator, was filtered, washed with absolute alcohol, ether, and finally dried in vacuo. No attempt was made to purify the substances obtained in these experiments because of the small amount isolated. One per cent solutions gave positive Molisch tests, faintly positive biuret tests, and negative trichloracetic acid, sulfosalicylic acid, and xanthoproteic acid tests.

The pH of each suspension before and after electrolysis was:

$NaCl$	– 7.4 before; 8.0 after electrolysis
$NaNO_3$	– 7.3 before; 3.4 after electrolysis
Na_2SO_4	– 7.5 before; 2.8 after electrolysis
$NaC_2H_3O_2$	– 7.7 before; 5.7 after electrolysis

With the exception of the NaCl electrolyte, the pH decreased after electrolysis.

These results may explain the views of Yen and Kurotchkin[4] that the liberation of specific polysaccharide substances during electrolysis in sodium chloride solution is attributable to three factors: (a) The sodium hypochlorite which is formed during electrolysis causes lysis of the organisms; (b) the sodium hydroxide which accumulates combines with bacterial protein; (c) the alkali proteinate is attracted to and precipitated at anode, leaving the specific carbo-

hydrate in solution. In the $NaNO_3$, Na_2SO_4, and $NaC_2H_3O_2$ solutions, probably only (b) and (c) are involved as no sodium hypochlorite is formed.

SUMMARY

A comparatively rapid method of preparing protein-free soluble specific substances of pneumococci is described. The substances obtained may be used in the routine standardization of antipneumococci sera. In our procedure, autolysis is replaced by subjecting a suspension of the organisms in NaCl solution (or in $NaNO_3$, $NaC_2H_3O_2$, or Na_2SO_4 solution) to an electric current. Most of the protein is precipitated during electrolysis. The type-specific capsular polysaccharide is isolated from the aqueous solution of the electrolysate by methods similar to those of Heidelberger, Kendall, and Scherp,[1] and Goebel et al.[2]

I wish to thank Mrs. H. Bucca and Miss A. Berlow for their assistance in the bacteriologic work required in this study.

REFERENCES

1. Heidelberger, M., Kendall, F. E., and Scherp, H. W.: The Specific Polysaccharides of Types I, II, and III Pneumococcus. A Revision of Methods and Data, J. Exper. Med. 64: 559, 1936.
2. Goebel, W. F., Beeson, P. B., and Hoagland, C. L.: Chemoimmunological Studies on the Soluble Specific Substance of Pneumococcus. IV. The Capsular Polysaccharide of Type XIV Pneumococcus, J. Biol. Chem. 129: 455, 1939.
3. Brown, Rachel: Chemical and Immunological Studies of the Pneumococcus. V. The Soluble Specific Substances of Types I-XXXII, J. Immunol. 37: 445, 1939.
4. Yen, A. C. H., and Kurotchkin, T. J.: Preparation of Specific Bacterial Carbohydrate Substances by Electrolysis, J. Infect. Dis. 56: 238, 1935.
5. Murao, K., and Morimoto, T.: Die Elektro-bakteriolytischen Immunogen; ueber die Bacteriolyse durch Elektrischen Strom, Nagasaki Igakkwai Zassi 13: 1401, 1935; Zentralbl. f. Bakt., Abt. I 137: 206, 1936.
6. Sevag, M. G.: Eine Neue Physikalische Enteiweissungsmethod zur Darstellung Biologisch Wirksamer Substanceen. Isolierung von Kohlenhydraten aus Huhnereiweiss und Pneumococcen, Biochem. Ztschr. 273: 419, 1934.

VENIPUNCTURE IN THE PRESENCE OF EDEMA*

STEVEN O. SCHWARTZ, M.D., CHICAGO, ILL.

CONSIDERABLE difficulty is often encountered in locating a vein for the purpose of venipuncture in the presence of marked edema involving the arms. The most common procedure in these cases has been to cut down on the vein.

The writer wishes to describe a simple method for which no priority is claimed, but which does not seem to be commonly known or employed. The technique is as follows: The patient's elbow is held by the physician in such a manner that the thumb is over the antecubital space. Pressure is exerted by the thumb for a period of from thirty to sixty seconds producing pitting. In the bottom of the resulting "pit" the antecubital veins stand out very prominently, since the edema fluid has been expressed not only from the overlying tissues but from the tissues surrounding the veins as well. So prominent are the veins made by this technique that the use of the tourniquet may occasionally be superfluous.

*From the Department of Medicine, Cook County Hospital.
Received for publication, February 25, 1943.

SIMPLIFIED KLOTZ-MACLACHLAN SOLUTION FOR TEMPORARY COLOR PRESERVATION*

BERT E. STOFER, M.D.
DETROIT, MICH.

AMONG the criteria for the ideal fixing fluid are color preservation, ease of preparation and use, rapid fixation of tissue, low cost, and permanency. It is, of course, difficult to find in a single fluid all these desirable properties. With the advent and popular acceptance of color photography the necessity for permanent preservation of specimens became lessened. A good colored lantern slide is a fine permanent record.

In many cases, where the services of a photographer are not immediately available, the specimen must be preserved for a few hours or days in as near a normal state as possible. At times, the specimen itself may be needed for a single staff conference, student group, or other occasions where a permanent mount is not required but natural appearance is desirable. Here color preservation, ease of preparation, and adequate fixation for future sectioning are the chief considerations.

By use of a suitable fixing fluid the specimen can be photographed, demonstrated, sectioned at leisure, and finally discarded or disposed of otherwise. With such a routine, storage space can be kept to a minimum.

*From the Department of Pathology Receiving Hospital, Detroit, Michigan.
Received for publication, March 3, 1943.

In our search for a solution to meet the requirements of this routine various combinations of the Klotz-MacLachlan[1] two-solution procedure were investigated. By modifying the concentration of some of the constituents we were able to develop one solution which has been satisfactorily used in a number of laboratories for more than one year. It fulfills the criteria of color preservation, ease of preparation, and adequate fixation. It is not prohibitively expensive.

The solution is made as follows:

Artificial carlsbad salt	487 Gm.
Chloral hydrate	175 Gm.
Formalin (40 per cent)	150 c.c.
Distilled water to make	19,000 c.c. (5 gallons)

The formula for artificial Carlsbad salt is five times the amount given by MacLachlan[2] but is otherwise unchanged. This is so reported for convenience in preparing the solution.

Sodium sulfate	110 Gm.
Sodium bicarbonate	100 Gm.
Sodium chloride	90 Gm.
Potassium nitrate	190 Gm.
Potassium sulfate	10 Gm.

Care must be used to add enough of the solution to the jar. At least three, and preferably five volumes of solution must be used for each volume of tissue. The specimen must be kept submerged, and it is our practice to overlay with cotton batting those which float. The concentration of the formalin must be watched, and if the color fades too rapidly 100 instead of 150 c.c. may be used in the formula, but this results in somewhat less adequate fixation. We have noted that the red colors, such as are seen in a superficial vascular area, are heightened for a few minutes or an hour after contact with the fluid. By photographing specimens with these features within the specified time, striking color photographs may be obtained.

Specimens may be preserved from seven to twenty-one days, and with certain types of tissue even longer. Fatty tissue keeps the least well. Cytologic detail is not quite so good as formalin fixation, especially if the blocks are cut after the first week or adequate fluid has not been used. The simplicity and color-preserving qualities, however, are ideal, and for temporary preservation this solution is well suited.

SUMMARY

A modification of Klotz's fixing fluid is suggested which temporarily preserves color, adequately fixes tissue, and avoids excessive technical procedure.

REFERENCES

1. Klotz, Oscar, and MacLachlan, W. W. G.: A Modified Jores' Method for the Preservation of Colors in Gross Specimens, Internat. Ass. Med. Mus. Bull. 5: 59, 1915.
2. MacLachlan, quoted by Kolmer, John A., and Boerner, Fred: Approved Laboratory Technique, ed. 3, New York, N. Y., and London, 1941, D. Appleton-Century Co.

USE OF THE SOUTH AFRICAN CLAWED FROG (XENOPUS) AS AN ASSAY ANIMAL FOR GONADOTROPIC HORMONES*

ABNER I. WEISMAN, M.D., AND CHRISTOPHER W. COATES
NEW YORK, N. Y.

IN 1930, Hogben[1] first showed that anterior pituitary lobe extracts could cause external deposition of ova in the mature female *Xenopus laevis* (South African clawed frog). Following this original observation, other investigators corroborated Hogben's finding, and the use of the female Xenopus as an assay animal for gonadotropic substances then became established in South Africa.

The Xenopus differs from the common variety of American frogs in that it constantly ovulates and accordingly carries the ova in its abdomen. The eggs are extruded in natural life only upon stimulation by the male of the species. The Xenopus has never been seen to extrude any eggs when kept segregated from males under laboratory conditions, unless injected by hormones. Thus, the animal is ever ready to expel its eggs upon proper stimulation. The external deposition of eggs has come to be accepted as a reliable manifestation of gonadotropic activity.

Having previously shown that egg extrusion could be attained by injection of extracts from the urine and the serum of pregnancy, a comparative study of the efficacy of true anterior lobe extracts seemed to be the next logical procedure. In our previous investigations[2, 3] it was demonstrated that deposition of eggs could be induced equally successfully by 100 rat units of chorionic gonadotropin of pregnancy urine and from 30 Cartland-Nelson units or 1,000 international units of equine gonadotropin derived from pregnant mares' serum. The Xenopus thus affords an excellent opportunity to evaluate the comparative gonadotropic activity of the various hormone extracts.

PROCEDURE

The Xenopus unit was based on the deposition of eggs within 6 to 18 hours after a dorsal subcutaneous injection of the hormone tested. The smallest amount of hormone which constantly caused extrusion was defined as one Xenopus unit. For each separate dosage of hormone to be tested two mature female Xenopus frogs were used. Each Xenopus was placed in a two-gallon tank filled one-third with water. Each tank was furnished with a half-inch wire mesh placed one inch above the bottom of the tank to prevent the possibility of the Xenopus eating any of its eggs.

Sixteen tanks were set up for eight different dosages of the powdered gonodatropic factor of sheep anterior pituitary gland.† Tanks A and A′ held

*From the Department of Gynecology of the Jewish Memorial Hospital, and the New York Zoological Society.

Aided by a grant from the New York Biologic Research Foundation.
Received for publication, March 30, 1943.

†The anterior pituitary gland extract used in this study is prepared by G. D. Searle & Co., Chicago, and is commercially known as Gonadophysin.

frogs injected with 5 rat units; the frogs in B and B′ received 10 rat units; those in C and C′ received injections of 15 rat units; D and D′, 25 rat units; E and E′, 50 rat units; F and F′, 75 rat units; G and G′, 100 rat units; H and H′, 200 rat units.

The animals were all injected through the dorsal lymph space and were kept at room temperature. They were examined periodically up to 18 hours after injection. After 18 hours it was noted that the animals in A, A′, B, B′, C, C′ had not reacted by egg extrusion, while the animals in D and D′ (25 rat units) showed definite evidence of egg deposits. Similarly, the animals in the other tanks that received larger doses also reacted positively.

This entire experiment was repeated the following week with similar results being obtained.

RESULTS

From the data compiled in Table I it will be seen that anterior pituitary gland extracts of sheep are highly gonadotropic in the female Xenopus. Quantitatively, 25 rat units were sufficient to produce external deposits of eggs in these animals. On the basis of this and our previous findings, it must be concluded that even though pregnant mare's serum and pregnancy urine extracts do cause deposition of eggs, true pituitary extracts are more efficacious in producing this reaction and with smaller doses.

TABLE I

MINIMAL AMOUNTS OF GONADOTROPINS WHICH ARE CAPABLE OF INDUCING EGG EXTRUSION IN THE FEMALE XENOPUS LAEVIS

CHORIONIC GONADOTROPINS DERIVED FROM PREGNANCY URINE (2)		CHORIONIC GONADOTROPINS DERIVED FROM PREGNANT MARES' SERUM (3)		GONADOTROPINS DERIVED FROM ANTERIOR PITUITARY LOBE OF SHEEP	
Antuitrin-S	100 rat units[1]	Gonadogen	30 Cartland-Nelson units[3]	Gonadophysin	25 rat units[5]
Follutein	100 international units[2]	Anteron	1000 international units[4]		

[1]Chorionic gonadotropin from human pregnancy urine, Parke, Davis & Co., Detroit, Mich.
[2]Chorionic gonadotropin from human pregnancy urine, E. R. Squibb & Sons, New Brunswick, N. J.
[3]Equine gonadotropin, Upjohn Co., Kalamazoo, Mich.
[4]Equine gonadotropin, Schering Corp., Bloomfield, N. J.
[5]Anterior pituitary lobe extract of sheep, G. D. Searle & Co., Chicago, Ill.

Since the Xenopus was found to be so accurate in evaluating the activity of the gonadotropes, the animal has been utilized clinically in the assay of urine. Urines from normal nonpregnant women, pregnant women, patients in the postmenopausal state have been assayed by means of the Xenopus. A Xenopus unit is equivalent to that amount of gonadotropic substance which will cause external egg extrusion in two Xenopus within 6 to 18 hours. The results of our clinical assays are of such interest that they will be published separately in a subsequent publication.

SUMMARY

Extracts of the anterior pituitary gland of sheep when injected into the South African Clawed Frog (Xenopus) were capable of inducing ovulation and external extrusion of eggs with doses as small as 25 rat units. Similar

gonadotropic activity with other extracts are evaluated. The use of the Xenopus frogs for assaying the urine of patients for gonadotropic substances is suggested.

REFERENCES

1. Hogben, L.: Action of Anterior Pituitary Lobe Extracts on Egg Extrusion in the Female Xenopus, Proc. Roy. Soc. South Africa 5: 19, 1930.
2. Weisman, A. I., and Coates, C. W.: The Frog Test (*Xenopus laevis*), as a rapid diagnostic Test for Early Pregnancy, Endocrinology 28: 141, 1941.
3. Weisman, A. I., Snyder, A. F., and Coates, C. W.: Physiology of Egg Extrusion in Female Xenopus Frog Test for Pregnancy, Endocrinology 31: 323, 1942.

CHEMICAL

ESTIMATION OF SERUM GLOBULIN BY MEASUREMENT OF VISCOSITY OF FORMALIN-TREATED SERUM: PROTEIN PARTITION BY PHYSICAL METHODS*

SAWYER FOSTER, M.D., AND FERNANDO BIGURIA, M.D., BOSTON, MASS.

WITH THE TECHNICAL ASSISTANCE OF MISS MARGARET A. ADAMS, A.B.

INTRODUCTION

SINCE serum proteins are often altered in the course of disease, information concerning their total concentration and the relative proportions of albumin and globulin is important for diagnostic and therapeutic purposes. One of the most widely used methods for obtaining these data is the micro-Kjeldahl chemical determination of nitrogen,[1] in conjunction with the modified Howe's method of separation of albumin and globulin by sodium sulfate.[2] This is a time-consuming and elaborate procedure requiring skilled training.

The falling drop technique devised by Kagan,[3,4] employing physical methods, has simplified the determination of the total protein of the serum. However, no methods comparable in simplicity for measuring the fractions of albumin or globulin appear to be available. The combined chemical and physical method advanced by Barbour[5] for albumin estimation is somewhat complicated; the usual tests for globulin such as the Takata-Ara, formol-gel, etc., are qualitative and indicate only the presence of hyperglobulinemia.

In a previous study[6] a modification of the formol-gel reaction was described for estimating the globulin content of sera in hyperglobulinemia. Confirming Napier's work, which demonstrated the specificity of the formol-gel test for globulin, it was observed that for a 5 per cent purified human albumin solution, the addition of small amounts of formaldehyde caused no change in viscosity. With reference to the blood serum, it was shown by direct measurements that the addition of formaldehyde to the serum increases its viscosity and that the degree of change corresponds to the concentration of globulin.

The modified formol-gel reaction[6] is employed in sera containing abnormally increased concentrations of globulin, which can be estimated quantitatively by the observation of a readily detected change in viscosity. This is determined on samples of serum placed in test tubes and then diluted progressively with physiologic saline and treated with formaldehyde. After standing for a period of twenty-four hours at room temperature, the tubes are shaken vigorously, and a degree of viscosity is noticed in one of the dilutions in which the air bubbles produced by the shaking do not rise to the surface, but remain

*From the First and Third (Tufts) Medical Services, Boston City Hospital, and the Department of Medicine, Tufts College Medical School.

Aided by a grant from the Charlton Research Fund, Tufts College Medical School.

Received for publication, May 17, 1943.

stationary, i. e., the "stationary bubble" end point. In less viscous dilutions the bubbles rise slowly, in more viscous dilutions gelification occurs. The "stationary bubble" end point corresponds to a globulin concentration of 3.1 Gm. per 100 c.c. as determined by chemical methods. From such observations the globulin content of a given serum may be calculated by the formula: Gm. per 100 c.c. globulin $= \frac{3.1}{y}$, where y represents the fraction of 1.0 c.c. of serum present in the dilution showing the above end point. Accordingly this method employs an indirect measurement of viscosity and is limited in its uses to cases of hyperglobulinemia.

In the present study the method has been extended to include all ranges of concentration of serum globulin. Direct measurements of the viscosity of formalin-treated sera were correlated with their globulin content. From these data a formula was derived for the determination of all ranges of concentrations of serum globulin. The final object was to estimate the total protein of the blood serum by the falling drop technique, the globulin by the measurement of the viscosity of formalin-treated serum, and the albumin by subtraction of one from the other. The results obtained by physical and chemical methods were compared.

PART I

DEVELOPMENT OF A FORMULA FOR THE CALCULATION OF THE GLOBULIN CONTENT FROM THE MEASUREMENT OF VISCOSITY OF FORMALIN-TREATED SERUM

A. *Viscosity Measurements on Serums With Hyperglobulinemia.*—Twenty samples of venous blood were taken during the fasting state from 19 patients with hyperglobulinemia. The blood was allowed to clot and the serum drawn off. For each serum a series of tubes was set up, each containing an accurately measured amount of serum ranging from 0.20 c.c. to 1.92 c.c. respectively and then diluted with physiologic saline to a final volume of 2.00 c.c. To each dilution was added 0.1 c.c. U.S.P. formaldehyde. After shaking, the tubes were allowed to stand at room temperature for twenty-four hours.

Depending upon their ability to flow easily through the viscosimeter, two to five dilutions from a series were chosen for direct observation of viscosity with reference to physiologic saline as unity. The viscosity was measured by observing the rate of flow of each dilution in an Ostwald pipette[7] of 2 c.c. capacity immersed in a water bath at a constant temperature of 25°C. The Ostwald pipette was allowed to stand in the water bath for ten minutes in order to reach 25° C. before the rate of flow was measured, and each measurement was repeated 3 times to obtain an average. The viscosity was determined by the formula $V = \frac{T_1 \times S.G.}{T_2}$ Where V = viscosity; T_1 = time of flow in seconds of formalin-treated dilution; T_2 = time of flow in seconds of physiologic saline; and S.G. = specific gravity of formalin-treated dilution. Since the specific gravity need be expressed to only two significant figures this factor becomes 1.0. A total of 66 dilutions were studied for their viscosity.

B. *Globulin Determinations.*—The globulin content of each serum in which the viscosity was measured in the above manner was determined in two ways:

(1) by the "stationary bubble" end point of the modified formol-gel reaction, and (2) by micro-Kjeldahl nitrogen analysis in conjunction with the modified Howe's method of separation of albumin and globulin. The globulin values. obtained by means of the "stationary bubble" end point, compare closely with the values found chemically with an average difference of 5 per cent and individual variations from 0 to 15 per cent (Table I). Since the results obtained by the "stationary bubble" end point could be reproduced more consistently, they were selected as the standard to calculate the globulin concentration of the dilutions prepared from each serum.

TABLE I

GLOBULIN VALUES, GM. PER 100 C.C. DETERMINED BY TWO METHODS

RESULTS	MODIFIED FORMOL-GEL REACTION "STATIONARY BUBBLE" END POINT	CHEMICAL $NaSO_4$ PRECIPITATION AND MICRO-KJELDAHL
1	3.5	3.49
2	3.9	3.99
3	3.7	3.74
4	4.3	3.80
	No. 4 repeated 2 days later	
5	3.9	3.66
6	3.8	3.75
7	4.0	4.18
8	3.1	3.13
9	5.7	5.87
10	4.8	5.20
11	4.4	4.06
12	4.3	4.01
13	3.7	4.03
14	4.0	4.05
15	3.7	3.57
16	3.1	3.63
	No. 16 repeated 2 days later	
17	3.1	3.25
18	3.1	3.37
19	3.4	3.43
20	4.1	4.28

C. *Derivation of Formula Expressing Globulin Concentration in Terms of Viscosity.*—The viscosity values of the 66 dilutions mentioned in Section A were related to their respective globulin values mentioned in Section B. By plotting the globulin values against the log. (viscosity — 1), a straight line was obtained when the globulin concentration of the dilutions was less than 2.5 Gm. per 100 c.c. Above this level the straight line did not persist. The equation of the straight line, determined by the customary method of least squares, was found to be G = 1.55 log. (V-1) + 1.64 where G is the grams of globulin per 100 c.c. and V is the viscosity. Fig. 1 shows all the data and the straight line in the region to which it may be applied.

PART II

APPLICATION OF THE FORMULA G = 1.55 LOG. (V-1) + 1.64 TO THE DETERMINATION OF ALL RANGES OF GLOBULIN IN BLOOD SERUM

Serum was obtained as before and two test tubes were prepared, one containing 2 c.c. of undiluted serum and the other, 1 c.c. of serum diluted with

1 c.c. of physiologic saline. To each was added 0.1 c.c. U.S.P. formaldehyde. The tubes were shaken, and, after a period of 24 hours at room temperature, observations of viscosity were performed as described above. The undiluted serum was used if it was capable of flowing freely through the Ostwald pipette. Otherwise the diluted serum was used. The viscosity reading was substituted in the formula G = 1.55 log. (V-1) + 1.64, and the globulin concentration multiplied by 2 when serum diluted 50 per cent was used. The total protein in each serum was determined by the falling drop technique of Kagan, and the albumin content was obtained by subtracting globulin values from the total protein.

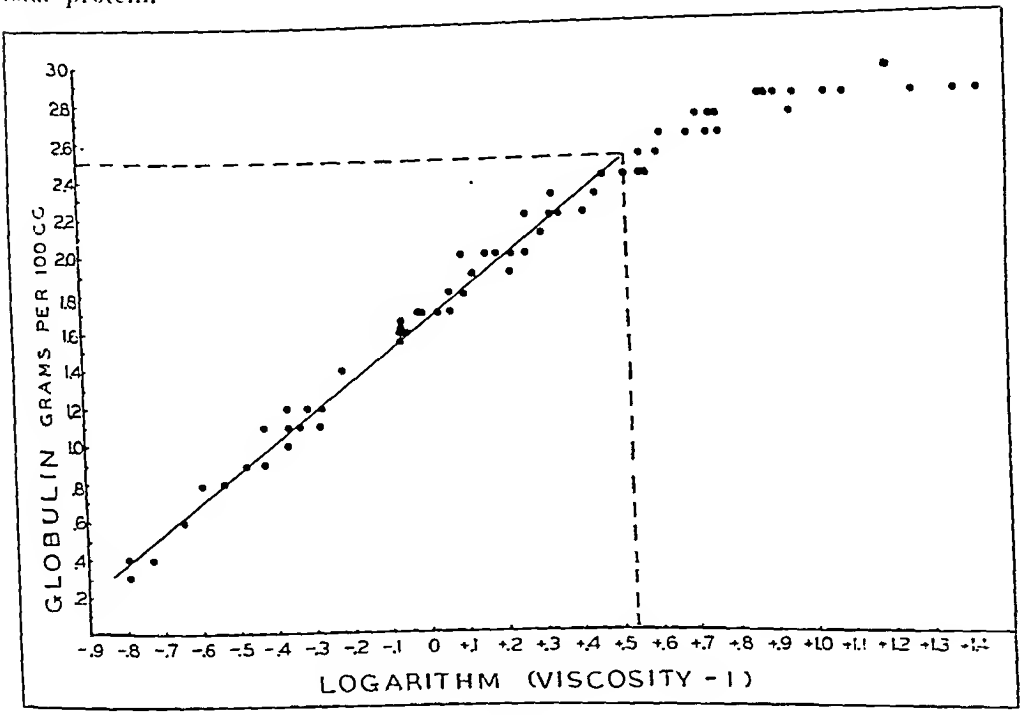


Fig. 1.

The total protein and the globulin and albumin content of 18 sera were determined. The comparative results between the chemical methods and the combined use of the falling drop and the viscosimetric methods are shown in Table II. The differences in globulin content between the chemical and the physical methods varied from 0 to 0.6 Gm. per 100 c.c. with an average of 0.2 Gm. per 100 c.c. and an average percentage difference of 7; the variations in the case of albumin were from 0 to 0.8 Gm. per 100 c.c. with an average difference of 0.2 Gm. per 100 c.c. and a percentage difference of 8. The total protein differences were from 0 to 0.4 Gm. per 100 c.c. with an average of 0.2 Gm. per 100 c.c. and a percentage difference of 4.

Experiments 11, 12, and 13 showed a variation not consistent with the general average. A search for the cause of such irregularity revealed that improper cleansing of the pipettes and especially of the Ostwald pipette was responsible for the abnormal results. When repeated with adequate precautions, the observations were consistent with the earlier determinations.

TABLE II

PROTEIN DETERMINATIONS. COMPARISON BETWEEN PHYSICAL AND CHEMICAL METHODS

		GM. PER 100 C.C.		DIFFERENCES	
		FALLING DROP AND VISCOSIMETRIC METHODS	CHEMICAL METHODS	GM. PER 100 C.C.	PER CENT
	T.P.	6.5	6.5	0	0
1	Glob.	1.9	1.8	+0.1	6
	Alb.	4.6	4.7	-0.1	2
		6.7	6.4	+0.3	5
2		2.0	1.7	+0.3	18
		4.7	4.7	0	0
		5.4	5.2	+0.2	4
3		3.1	3.1	0	0
		2.3	2.1	+0.2	10
		7.3	7.1	+0.2	3
4		2.7	2.6	+0.1	4
		4.6	4.5	+0.1	2
		5.6	5.4	+0.2	4
5		3.5	3.5	0	0
		2.1	1.9	+0.2	10
		6.0	5.6	+0.4	7
6		1.8	1.9	-0.1	5
		4.2	3.7	+0.5	13
		5.9	5.7	+0.2	4
7		3.1	3.4	-0.3	9
		2.8	2.3	+0.5	22
		5.2	5.2	0	0
8		1.7	1.7	0	0
		3.5	3.5	0	0
		6.4	6.5	-0.1	2
9		2.9	3.0	-0.1	3
		3.5	3.5	0	0
		5.2	5.3	-0.1	2
10		2.1	2.3	-0.2	9
		3.1	3.0	+0.1	3
		6.9	7.2	-0.3	4
11		3.0	2.5	+0.5	20
		3.9	4.7	-0.8	17
		5.6	5.3	+0.3	6
12		3.6	3.0	+0.6	20
		2.0	2.3	-0.3	13
		6.0	5.7	+0.3	6
13		4.5	4.1	+0.4	10
		1.5	1.6	-0.1	6
		7.0	6.6	+0.4	6
14		3.0	3.0	0	0
		4.0	3.6	+0.4	11
		5.6	5.6	0	0
15		2.7	2.9	-0.2	7
		2.9	2.7	+0.2	7
		8.2	7.8	+0.4	5
16		4.1	3.9	+0.2	5
		4.1	3.9	+0.2	5
		6.2	5.9	+0.3	5
17		3.9	3.9	0	0
		2.3	2.0	+0.3	15
		7.2	7.0	+0.2	3
18		3.4	3.3	+0.1	3
		3.8	3.7	+0.1	3

CONCLUSIONS

1. The addition of small amounts of formaldehyde to blood serum produces an increase in the viscosity of the serum. This change is due to globulin, since it does not occur when formaldehyde is added to a 5 per cent solution of purified human albumin.

2. A strictly linear relation was demonstrated between the degree of viscosity and the globulin content of formaldehyde-treated serum, when the globulin content was below 2.5 Gm. per 100 c.c. This relation is expressed by the equation $G = 1.55 \log. (V\text{-}1) + 1.64$ where G = grams of globulin per 100 c.c. and V = viscosity.

3. With this formula it is possible to estimate quantitatively the globulin concentration of the blood serum by measuring its viscosity after the addition of formaldehyde.

4. The value of the viscosimetric method using formaldehyde-treated serum for the estimation of globulin is enhanced when used together with the falling drop method for estimation of total protein. Through their combination the total protein, albumin, and globulin content of any serum can be estimated entirely by physical means with great saving of time, material, and number of personnel.

5. A comparison of the physical and chemical methods shows an average difference of 0.2 Gm. per 100 c.c. for total protein, albumin, and globulin. This error is similar to that inherent to the chemical method.

6. The careful observance of certain precautions must be emphasized: the equipment must be kept meticulously clean; the amounts of serum, saline, and formaldehyde should be measured with accuracy; the water bath must be kept at a constant temperature.

We gratefully acknowledge the assistance we have received from Drs. T. H. Ham and F. H. L. Taylor from the Thorndike Memorial Laboratory, and from Miss Jane Worcester, Associate in the Department of Vital Statistics, Harvard School of Public Health.

REFERENCES

1. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. Methods, Baltimore, 1932, Williams & Wilkins Co., p. 691.
2. Howe, Paul E.: The Use of Sodium Sulphate as the Globulin Precipitant in the Determination of Proteins in Blood, J. Biol. Chem. 49: 93, 1921.
3. Kagan, B. M.: A Simple Method of the Estimation of Total Protein Content of Plasma and Serum. I. A Falling Drop Method for the Determination of Specific Gravity, J. Clin. Investigation 17: 369, 1938.
4. Kagan, B. M.: A Simple Method for the Estimation of Total Protein Content of Human Plasma and Serum by the Use of the Falling Drop Method, J. Clin. Investigation 17: 373, 1938.
5. Barbour, P. H., Jr.: The Application of the Falling Drop Method for Specific Gravity Measurement to the Determination of Serum Albumin, Yale J. Biol. and Med. 14: 107, 1941.
6. Biguria, F., and Foster, S.: The Formol-Gel Reaction, J. Lab. & Clin. Med. 26: 1211, 1941.
7. Findlay, A.: Physical Chemistry for Students of Medicine, London, 1927, Longmans, Green and Co., p. 27.

A METHOD FOR EXACT PROTEIN DETERMINATION IN SMALL QUANTITIES OF SPINAL FLUID*

F. RAPPAPORT, PH.D., AND E. LASOWSKI, PETAH TIQVA, PALESTINE

ALTHOUGH the knowledge of the exact protein content of the spinal fluid is frequently of diagnostic importance, the present methods when carried out with small quantities of fluid yield results which may show an error as high as 100 per cent.[1] Exact results may, of course, be obtained with the old Kjeldahl method, but this requires such large quantities of fluid that they cannot as a rule be obtained, or only with difficulty.

PRINCIPLE OF THE NEW METHOD

The total proteins of the spinal fluid are precipitated with phosphomolybdic acid, centrifugated, and the sediment, after being dissolved in diluted sodium hydroxide, is quantitatively carried over to a Kjeldahl flask. The further procedures are those of nonprotein nitrogen determinations.[2, 3]

REAGENTS

1. For protein precipitation:

(a) Dissolve 5 Gm. of anhydrous sodium sulfate and 8 Gm. of phosphomolybdic acid in 200 c.c. distilled water.

Add 20 c.c. of 5N sodium hydroxide, and boil the mixture for one-half hour. After cooling, add 10.6 c.c. of concentrated sulfuric acid and water up to 1000 c.c.

(b) A 4 per cent solution of chemically pure sodium hydroxide.

2. For digestion:

Dissolve 1 Gm. of phosphomolybdic acid in about 50 c.c. of water. Add 1 c.c. of Kjeldahl hydroxide and boil for twenty minutes to drive off the possibly present ammonia; add 30 c.c. of concentrated sulfuric acid and 5 c.c. of syrupy phosphoric acid. Add water to make up 100 c.c.

3. For neutralization:

(a) A 27 per cent solution of chemically pure sodium hydroxide.

(b) Dissolve 15 mg. of methyl red and 60 mg. of thymol blue in 20 c.c. of N/1 NaOH and add water to 1000 c.c.

(c) A saturated (about 5 per cent.) solution of sodium fluoride.

To make this reagent (3) mix 6 parts of solution a with 1 part of solution b and 3 parts of solution c. This mixture keeps indefinitely.

4. For titration:

(a) Buffer solution. Dissolve 84.5 Gm. boric acid and 15.6 Gm. sodium hydroxide in about 800 c.c. water. Boil vigorously for thirty minutes to remove NH_3. Cool and add water to 1000 c.c.

*From the Biochemical Laboratory of the Beilinson Hospital, Petah Tiqva, Palestine.
Received for publication, April 8, 1943.

(b) Dissolve 20 Gm. of sodium bromide in a little water in a measuring flask. Add 8 Gm. elemental bromine and water to 1000 c.c. Just before using pour 10 c.c. of solution (b) into a 50 c.c. measuring flask. Add 25 c.c. of buffer solution and water to 50 c.c.

(c) Potassium iodide in granules.

(d) Fuming hydrochloric acid diluted with an equal quantity of water.

(e) 0.25 per cent starch solution.

(f) N/100 solution of sodium thiosulfate.

PROCEDURE

Place in a small 5 by 50 m.m. test tube (Widal tubes are best) exactly 1 c.c. of the spinal fluid. If the Pandy reaction is positive, only 0.5 c.c. of fluid is used. Add 2 c.c. of phosphomolybdic acid reagent (1a) to precipitate the proteins. Place in water bath at 56° C. for a few minutes; then centrifuge for about ten minutes at 3,000 revolutions. Carefully pour off the supernatant fluid and dissolve the sediment in 0.3 to 0.5 c.c. of 4 per cent sodium hydroxide (1b). Draw up the contents of the tube with great care, using a serologic 1 $cm._3$ pipette: and place them into a Kjeldahl flask which contains 2 c.c. of the digestion mixture reagent (2) and a few glass pearls. Again fill the tube with some water, and with the same serologic pipette transfer it to the Kjeldahl flask. Repeat this a number of times drawing up and blowing out the contents of the pipette and washing the sides of the tube. Set up two Kjeldahl flasks with the fluid to be examined and two flasks filled only with the phosphomolybdic acid reagent as blanks. Boil both series with the digestion reagent until the contents of the flasks become colorless and fumes no longer arise. After cooling the flasks, dilute the contents of each with 2 to 3 c.c. of water, and while continuously cooling with water, add, drop by drop, the mixed indicator, NaOH, sodium fluoride solution (3) until it turns blue: all flasks require about the same number of drops. Neutralize, therefore, all the flasks in the series in this manner; then add to each 5 c.c. of the hypobromine solution (Reagent 4b mixed with buffer solution 4A for use). Then add to each a few granules of potassium iodide (4c) and 2 to 3 c.c. of diluted hydrochloric acid (4d). Titrate with N/100 thiosulfate (4f) solution until the mixture is straw yellow. Add starch and continue titration until the colorless end point is reached.

Note: We use for our determinations micro-Kjeldahl flasks whose dimensions are: length of flask, 135 mm.; diameter of neck, 20 mm.; length of neck, 85 mm.; diameter of body of flask, 40 mm.; volume of flask, about 35 c.c.

CALCULATION

1 c.c. of N/100 thiosulfate equals 0.046 mg. nitrogen, which multiplied by the protein number 6.25 corresponds to 0.2875 mg. of protein. This figure multiplied by 100 gives the protein content as mg. per 100 c.c. Example: 2.1 c.c. thiosulfate for 1 c.c. of spinal fluid was used. Calculation: $2.1 \times 0.2875 \times 100 = 60.375$ mg. protein per 100 c.c. of fluid.

The quantity of thiosulfate used is calculated by subtracting the quantity used in the unknown from that in the blank.

The application of this method for the determination of albumin, globulin, and nonprotein nitrogen in spinal fluid will be discussed in another communica-

tion. We shall also report our findings on the protein content of normal spinal fluid as determined by this method.

CONCLUSION

A method is described by which exact protein determinations are possible even in very small quantities of spinal fluid.

REFERENCES

1. Kingsbury, F. B., Clark, C. P., Williams, Gertrude, and Post, Anna L.: The rapid determination of albumin in urine, J. Lab. & Clin. Med. 11: 981, 1926.
2. Rappaport, F.: Verfeinerung der massanalytischen Bestimmung des Rest-N im Blute ohne Destillation, Klin. Wchnschr. 16: 1190, 1937.
3. Rappaport, F.: Mikrochemie des Blutes, Wien-Leipzig, 1935, ed. 1, *Emil Haim*, Editor, p. 86.

A HISTOCHEMICAL METHOD FOR DEMONSTRATING THE PRESENCE OF SULFONAMIDES IN THE TISSUES*

George M. MacKee, M.D., Franz Herrmann, M.D., Rudolf L. Baer, M.D., and Marion B. Sulzberger, M.D., New York, N. Y.

A RECENT communication[1] reported the development of new vehicles ("Penetrasols"), which increased the penetration of a large variety of substances through grossly intact skin (for example, "protein" allergens, iron, mercury, bismuth, and the sulfonamides). The penetration of externally applied sulfonamides was demonstrated by the presence of these drugs in the blood and urine. Further evidence of the penetration of sulfonamides into and through the skin, was adduced by biopsy of the skin area and the use of a newly developed specific histochemical color reaction with the sulfonamide.

We report here the details of our method, which we believe may find many other applications. Preliminary report has appeared.[2]

The vehicles studied were of different compositions, but in each instance equal quantities of the vehicle, containing equimolecular concentrations of the drug, were gently rubbed in, in identical manner to skin areas of identical size. The only preceding preparation consisted of clipping the hairs of the guinea pigs as short as possible with a sharp pair of scissors.

General ether anesthesia was used in the guinea pigs; and local anesthesia with procaine in man. A type of block anesthesia was employed through injection of the anesthetic around and well away from the site of excision to eliminate all possible procaine contamination of the excised tissue. This is an indispensable precaution, as the presence of procaine, or even of injected water, could produce erroneous results. The line of excision must be kept at a considerable distance from the site of inunction to avoid displacement of the inunction material with the scalpel. Each excision was performed after a given time had elapsed following inunction.

*From the Skin and Cancer Unit, New York Post Graduate Medical School and Hospital, Columbia University.

This research was made possible by a grant from the Wallace Laboratories, Inc., New Brunswick, N. J.

Received for publication, June 4, 1943.

PROCEDURE OF TISSUE EXAMINATION

A. *Fixation.*—No liquid fixatives could be used to obtain a *histotopical* demonstration, i.e., a demonstration of the drug's localization and distribution within tissue, because it was evident that significant quantities of sulfonamide might be removed or displaced through transportation with the liquids. To overcome these difficulties, we used a gaseous, instead of a liquid, fixative.* Dry formaldehyde gas was chosen because the use of osmic acid fumes would have obscured our findings. Fixation was carried out as follows: Trioxymethylene (paraformaldehyde) powder is spread in about 1 cm. depth over the bottom of small glass beakers (1 to 4) which are placed within a glass jar and their bases surrounded by 1 cm in depth of powdered trioxymethylene. The jar must be equipped with a well-fitting glass cover (an ordinary butter jar, 4 × 4 × 3 inches, will do). The small beakers are roofed, either with a piece of gauze fixed by a rubber band, or with a small glass lattice. The biopsy specimens are placed flatly on these "sieves." Each beaker is clearly marked to keep a record of the specimens. After the small beakers with their fresh specimens have been placed within the larger jar, the latter is covered and kept closed throughout the experiment by several windings of adhesive tape around the line of closure.

The jars are kept at room temperature and fixation takes place within two to twenty-four hours, depending on the size and thickness of the specimens. The most favorable moment for microtome cutting is immediately after fixation, because some shrinkage and brownish discoloration gradually occur; but, when necessary, satisfactory sections can be obtained even after ten to fourteen days.

B. *Cutting of Sections.*—After fixation, frozen sections of 10 to 20 μ are cut. As our particular purposes necessitated comparable specimens, the sections used were all made of approximately equal thickness (about 16 μ).

In the study of skin penetration, as already stressed by Fuerbringer[3] and later on by others (Zwick, Eller and Wolff, and Duemling[4]), the specimen must be kept in such a position on the freezing table that the epidermal surface is kept toward the operator, never directed toward the knife. This tends to obviate the possibility of carrying material from the epidermal surface into the deeper structures with the movement of the knife. In cutting thin and hairy animal skin, the epidermal part often tends to separate as a thin lamella. This can be overcome by placing the specimen sagittally, at an angle of about 90 degrees to the edge of the knife. Here again, the epidermal surface must be turned away from the knife while cutting.

C. *Chemical Process and Reagents Used.*—The demonstration of sulfonamide within the tissue is based on the formation of a characteristic precipitation. Identification by means of formation of sulfonamide crystals, as carried out with a solution of iodine and sodium iodide, etc., in microchemical analysis[5] is not feasible in histotopical studies because the drug would be displaced from its original site. Similarly Marshall's reagent, commonly used for quantitative sulfonamide analysis, cannot be employed because extraction would occur with the diazotation and with the reaction.

"Ehrlich's Reagent." p-dimethylaminobenzaldehyde, as used by Werner and others[6, 7, 8] for quantitative sulfonamide analysis in blood, urine, and

*For the same reasons we found these technical precautions necessary in other histotopical examinations; i.e., in our studies on the penetration of Fe., Bi., Hg. etc., through the skin.

other biologic fluids, proved most suitable for our purposes. Extensive preliminary trials in vitro and in tissue sections, with solutions of this reagent in water containing various concentrations of mineral acids in acid alcohol, etc., have shown optimal precipitation with the following solution:

p-dimethylaminobenzaldehyde	1.0 Gm.
Alcohol abs.	95.0 c.c.
Concentrated HCl	5.0 c.c.

The purest obtainable aldehyde, having little, or at most a light buff color when dry, is used. The solution is kept in an amber-colored flask, well closed by a ground glass stopper. The liquid gradually becomes yellowish, and it is preferable to use fresh solutions (never more than 2- to 3-weeks-old).

This reagent yields a lemon-yellow precipitate with low concentrations of free sulfonamides and an orange shade with higher concentrations of the drugs. The reacting group of the sulfonamides is their free "aniline," NH_2 group. Because the colored reaction product bleaches out and gradually assumes other shades on exposure to the air, in vitro as well as in the tissue, the sections cannot be made to keep the characteristic color for long. Extensive studies attempting to defer the fading time and to avoid any procedures or admixtures which might tend to produce variation in color have resulted in the adoption of the following routine technique which we have carried out satisfactorily for the past two years on a great number of specimens of human and animal skin.

The sections (16 μ), obtained as described above, are placed *directly* on a clean slide, with a dry brush (camels' hair). Two drops of the p-dimethylaminobenzaldehyde solution in acid alcohol (see above) are dropped on the section. After three to five minutes, 1 to 2 drops of absolute alcohol containing 5 per cent of concentrated HCl are added (1 drop for the smaller and 2 drops for the larger sections). The sections are dried immediately by careful absorption of excess fluid with small pieces of filter paper, or before a rotating fan, or by both methods. (Careful studies under controlled conditions ruled out the possibility that the removal of the acid-alcohol with filter paper could cause displacement of the colored product in the section.) Warming is not advisable. As soon as dry, the sections are covered with the following concentration of dissolved Damar resin: Damar resin 10.0 Gm., Xylene 10.0 c.c. (This "thick" mounting medium has proved superior to Canada balsam, cedar oil, origanum oil, albumen-glycerin, etc., which either extract the colored product, or produce color changes, or lead to quick fading.) Then the cover slip is sealed with liquid paraffin melting at 56° C. Because exposure to air impairs the intensity of the color, after the addition of the acid alcohol the whole procedure must be accomplished as quickly as possible and air bubbles must be removed from under the cover slip. The successive use of acid alcohol and the solution of Damar resin in xylene has the additional advantage of producing a satisfactory and rapid clearing of the sections. Microscopic examination must be as early as possible; and colored photos, if desired, should be taken not later than three to four hours after the reaction has occurred.

As stated, the presence of sulfonamide is characterized by a bright orange or lemon-yellow color. Counterstaining has not proved valuable because the reaction color of the sulfonamide tends to be obscured. Black and white photo-

graphs may show the sulfonamide picture and distribution when the tissues themselves do not contain pigment or other material darker in shade than the orange-yellow sulfonamide reaction.

Figs. 1, 2, 3, and 4 are black and white photographs which were retaken from colored diapositives and which show the different routes of penetration of sulfonamides through the skin of *the same* albino guinea pig after inunction of sulfonamide under strictly comparable conditions, but dissolved in 3 different types of vehicles. The photographs are presented here merely to show the technical possibilities of our method in differentiating various types of distribution in the tissues. (Details on composition of the different vehicles and mode of application, etc., will be the subject of forthcoming communications.)

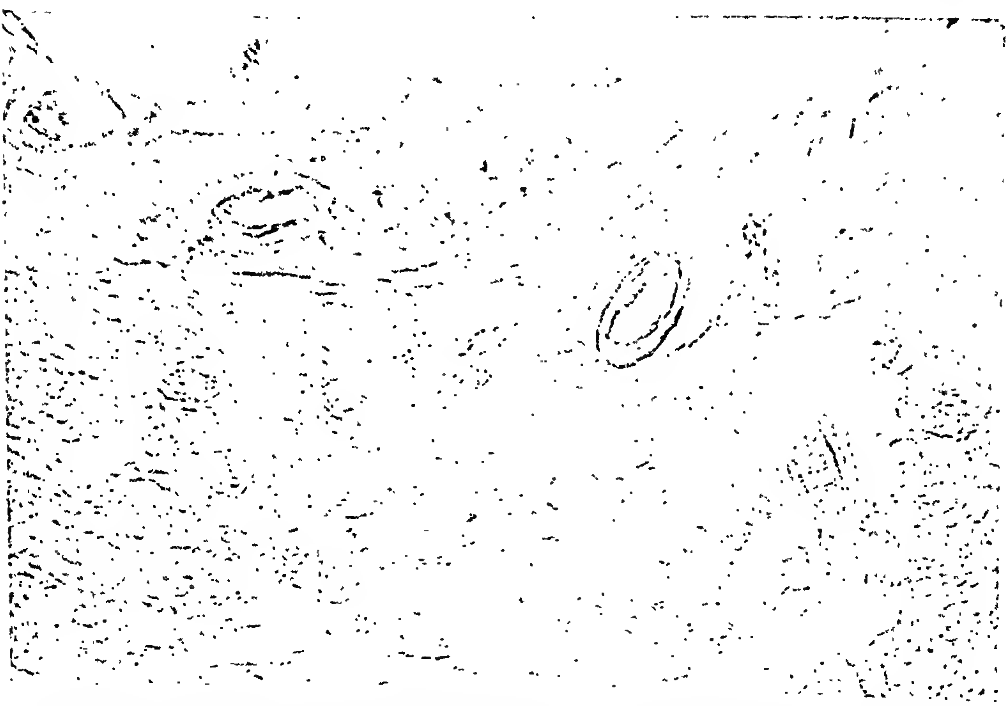


Fig. 1.—Control: Area of skin which received no preceding inunction but which was excised and histologically prepared and treated with p-dimethylaminobenzaldehyde in acid-alcohol exactly like the other sections shown in Figs. 2 to 4. No reaction seen.

Figs. 1 to 4.—Figures are all of specimens of biopsies taken from different skin areas of the same albino guinea pig. (Black and white reproductions of colored diapositives). (Particulars on the composition of the penetrating vehicles designed to achieve the different effects will appear in a future publication.)

D. *Controls.*—Several controls are essential to make as certain as possible that the yellow to orange color observed in the sections is actually due to a sulfonamide reaction and not to the presence of preformed yellowish material or pigments (e.g., melanin, blood pigments, carotene, bile, etc.).

Control 1: Examination of several sections from the same tissue specimen processed in identical fashion as the sections treated with p-dimethylamino-benzaldehyde, but *not* exposed to this reagent. Comparative examination of these sections either shows that preformed colored matter is absent, or reveals the site of any preformed colored matter of a shade similar to that of the sulfonamide reaction product. As a rule, skin sections show little or no mate-

other biologic fluids, proved most suitable for our purposes. Extensive preliminary trials in vitro and in tissue sections, with solutions of this reagent in water containing various concentrations of mineral acids in acid alcohol, etc., have shown optimal precipitation with the following solution:

p-dimethylaminobenzaldehyde	1.0 Gm.
Alcohol abs.	95.0 c.c.
Concentrated HCl	5.0 c.c.

The purest obtainable aldehyde, having little, or at most a light buff color when dry, is used. The solution is kept in an amber-colored flask, well closed by a ground glass stopper. The liquid gradually becomes yellowish, and it is preferable to use fresh solutions (never more than 2- to 3-weeks-old).

This reagent yields a lemon-yellow precipitate with low concentrations of free sulfonamides and an orange shade with higher concentrations of the drugs. The reacting group of the sulfonamides is their free "aniline," NH_2 group. Because the colored reaction product bleaches out and gradually assumes other shades on exposure to the air, in vitro as well as in the tissue, the sections cannot be made to keep the characteristic color for long. Extensive studies attempting to defer the fading time and to avoid any procedures or admixtures which might tend to produce variation in color have resulted in the adoption of the following routine technique which we have carried out satisfactorily for the past two years on a great number of specimens of human and animal skin.

The sections (16 μ), obtained as described above, are placed *directly* on a clean slide, with a dry brush (camels' hair). Two drops of the p-dimethylaminobenzaldehyde solution in acid alcohol (see above) are dropped on the section. After three to five minutes, 1 to 2 drops of absolute alcohol containing 5 per cent of concentrated HCl are added (1 drop for the smaller and 2 drops for the larger sections). The sections are dried immediately by careful absorption of excess fluid with small pieces of filter paper, or before a rotating fan, or by both methods. (Careful studies under controlled conditions ruled out the possibility that the removal of the acid-alcohol with filter paper could cause displacement of the colored product in the section.) Warming is not advisable. As soon as dry, the sections are covered with the following concentration of dissolved Damar resin: Damar resin 10.0 Gm., Xylene 10.0 c.c. (This "thick" mounting medium has proved superior to Canada balsam, cedar oil, origanum oil, albumen-glycerin, etc., which either extract the colored product, or produce color changes, or lead to quick fading.) Then the cover slip is sealed with liquid paraffin melting at 56° C. Because exposure to air impairs the intensity of the color, after the addition of the acid alcohol the whole procedure must be accomplished as quickly as possible and air bubbles must be removed from under the cover slip. The successive use of acid alcohol and the solution of Damar resin in xylene has the additional advantage of producing a satisfactory and rapid clearing of the sections. Microscopic examination must be as early as possible; and colored photos, if desired, should be taken not later than three to four hours after the reaction has occurred.

As stated, the presence of sulfonamide is characterized by a bright orange or lemon-yellow color. Counterstaining has not proved valuable because the reaction color of the sulfonamide tends to be obscured. Black and white photo-

graphs may show the sulfonamide picture and distribution when the tissues themselves do not contain pigment or other material darker in shade than the orange-yellow sulfonamide reaction.

Figs. 1, 2, 3, and 4 are black and white photographs which were retaken from colored diapositives and which show the different routes of penetration of sulfonamides through the skin of *the same* albino guinea pig after inunction of sulfonamide under strictly comparable conditions, but dissolved in 3 different types of vehicles. The photographs are presented here merely to show the technical possibilities of our method in differentiating various types of distribution in the tissues. (Details on composition of the different vehicles and mode of application, etc., will be the subject of forthcoming communications.)

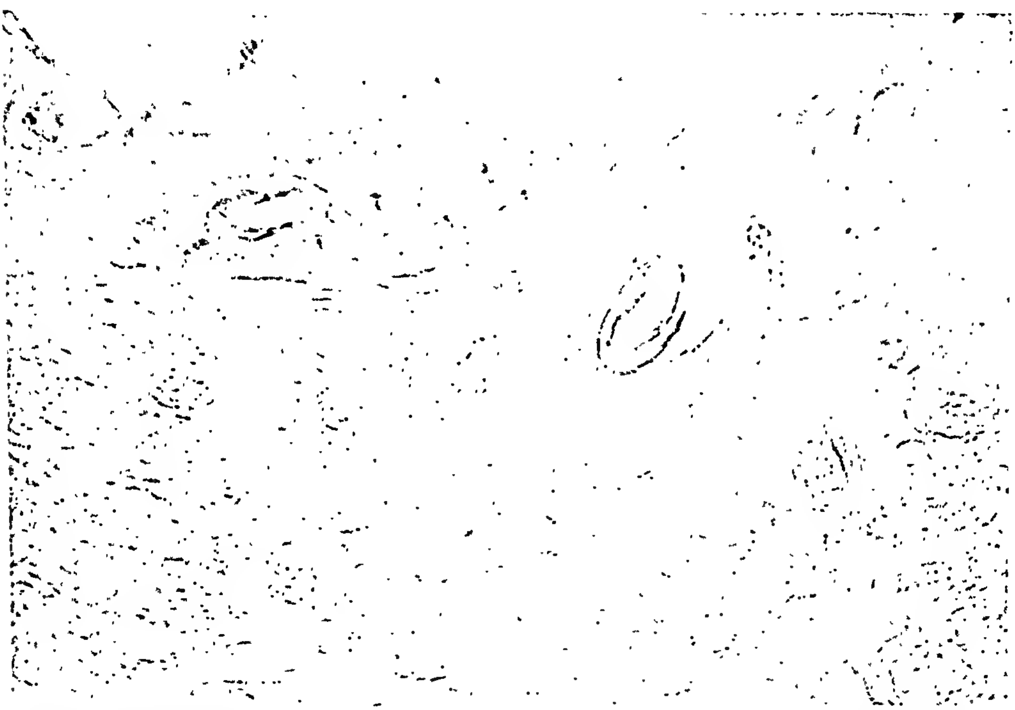


Fig. 1.—Control: Area of skin which received no preceding inunction but which was excised and histologically prepared and treated with p-dimethylaminobenzaldehyde in acid-alcohol exactly like the other sections shown in Figs. 2 to 4. No reaction seen.

Figs. 1 to 4.—Figures are all of specimens of biopsies taken from different skin areas of the same albino guinea pig. (Black and white reproductions of colored diapositives). (Particulars on the composition of the penetrating vehicles designed to achieve the different effects will appear in a future publication.)

D. *Controls.*—Several controls are essential to make as certain as possible that the yellow to orange color observed in the sections is actually due to a sulfonamide reaction and not to the presence of preformed yellowish material or pigments (e.g., melanin, blood pigments, carotene, bile, etc.).

Control 1: Examination of several sections from the same tissue specimen processed in identical fashion as the sections treated with p-dimethylamino-benzaldehyde, but *not* exposed to this reagent. Comparative examination of these sections either shows that preformed colored matter is absent, or reveals the site of any preformed colored matter of a shade similar to that of the sulfonamide reaction product. As a rule, skin sections show little or no mate-

rials in the color range of the sulfonamide reaction; and, if present, their color is duller and more brownish than that of the specific sulfonamide product, which, as stated, is characterized by its brightness.

Fig. 2.—Area of skin which received inunction of 10 per cent sulfonamide, applied in a vehicle consisting of lanolin and water. (Excision sixty minutes after inunction; fixation and treatment as described in text.) Note that the reaction product (darker areas) is practically confined to the surface and the upper parts of the horny layer.

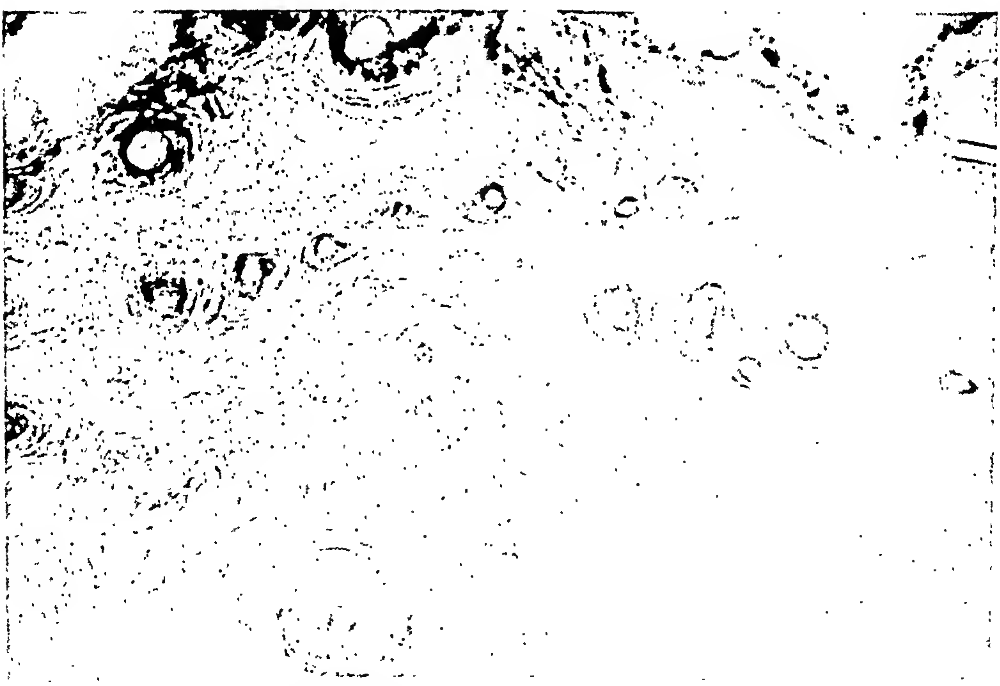


Fig. 3.—Area of skin which received inunction of 10 per cent sulfonamide dissolved in a penetrasol type of vehicle which favors penetration into horny structures. (Excision sixty minutes after inunction; fixation and treatment as described in text.)

(Note that the horny layer and the follicle cross sections show heavy deposits of the dark material, while other parts are relatively free.)

Control 2: Comparative examination of a specimen of the same or corresponding organ but obtained from a site known to be *free of sulfonamide.* Control biopsy specimens taken from the same individual at a skin area distant from that of the sulfonamide application, are fixed, cut, and treated with

p-dimethylaminobenzaldehyde, exactly as described for the demonstration of sulfonamides in the tissue. This is necessary to rule out the possibility of a "nonspecific" reaction. While these controls were uniformly negative (Fig. 1), it must be borne in mind that p-dimethylaminobenzaldehyde produces a yellow reaction with any primary aromatic amine, with urobilinogen, with higher concentrations of urea, and perhaps also with decomposition products of the tissue. We have sometimes observed this phenomena on merely warming a section. (Thus, as stated, warming must not be used in the drying procedure.) Furthermore, certain drugs other than sulfonamides (e.g., phenacetin, acetanilid) give reactions similar to those produced with sulfonamides. Procaine and related local anesthetics may also produce this effect; therefore, as already mentioned, no such solutions should be used to infiltrate the actual site of biopsy.

Fig. 4.—Area of skin which received inunction of 10 per cent sulfonamide in a penetrasol type of vehicle which favors penetration of all cutaneous structures. (Excision sixty minutes after inunction; fixation and treatment as described in text.) (Note that the reaction product [dark material] is found in all structures, including cutis.)

When all precautions have been rigorously observed, the described histotopical method is probably applicable to a large variety of tissues (urethra, other mucous membranes, tonsils, bone marrow, wounds, etc.), but is not likely to prove applicable to certain other tissues (liver, gall bladder, etc.). As is evident, the investigator should not rely upon an *absolute* accuracy of localization; however, when identical experiments *repeatedly* show the *same* localizations of the characteristic colored material; and, conversely, when deliberate experimental modifications *repeatedly* show characteristic *differences* in the localization of the colored material, the findings may be considered as elucidating

particulars of the drug's penetration, route, and distribution within the tissue structures. (See Figs. 2, 3, and 4.)

E. *Discussion.*—A few general comments should be made on our histochemical procedure itself and how we came to adopt the various steps finally selected.

As shown by Morris in reporting on sulfonamide determination in body fluids,[7] the compound responsible for the color is p-dimethylaminobenzylidene-p-aminobenzene sulfonamide. While Morris rightly stressed the dependence of the color on the pH, addition of a buffer is not necessary in our procedure, which is carried out almost in the absence of water. The HCl concentration used is so high that variations which may be caused by pH differences in the specimens are negligible.

In order to minimize the possibility of dissolving or moving parts of the reaction product, our sections are not immersed in the reagent solution, but are treated with the reagent directly while on the slide. On the other hand, the plain acid (HCl)-alcohol is added as a rinse, to ensure the removal of excess reagent and of other loose yellow or orange-colored matter. This rinsing unavoidably involves the loss of a fraction of the reaction product, but we believe this disadvantage to be more than offset by the advantage of confining the colored reaction product as strictly as possible to the original actual sites of the drug within the tissues. Our results are, therefore, more inclined to miss some of the sulfonamide, rather than to indicate sulfonamide where none was present in the tissues.

As already pointed out, the characteristic picture is not preserved for long, despite all available technical precautions to prevent fading. While undeniably inconvenient, the lack of stability of the color is further proof of the nature of the colored product and indicates that the yellow or orange material is produced by the chemical reaction with the sulfonamide and that it is not due to the presence of other or preformed colored matter, such as pigment, lipids, carotene, etc.

SUMMARY

A new method for histochemical and histotopical identification of sulfonamides in tissue sections is described and discussed. This method has been employed and found satisfactory in numerous studies on the percutaneous penetration of sulfonamides, after their solution in penetrating vehicles and their external application to grossly intact human and animal skins.

REFERENCES

1. Herrmann, F., Sulzberger, M. B., and Baer, R. L.: New Penetrating Vehicles and Solvents, Science 96: 451, 1942.
2. MacKee, G. M., Herrmann, F., Baer, R. L., and Sulzberger, M. B.: Demonstrating the Presence of Sulfonamides in the Tissues, Science 98: 66, 1943. (Preliminary Report of the Present Studies.)
3. Fuerbringer, P.: Experimentelle Untersuchungen über die Resorption und Wirkung des regulinischen Quecksilbers der grauen Salbe, Arch. f. path. Anat. u. Physiol. 82: 491, 1880.
4a. Zwick, K.: A Microscopic Study of Mercury Absorption from the Skin, J. A. M. A. 83: 1821, 1924.
b. Eller, Jos. J., and Wolff, S.: Permeability and Absorptivity of the Skin, Arch. Dermat. & Syph. 40: 900, 1939.
c. Duemling, W. W.: Wetting Agents—New Synthetic Chemicals of Use in Finer and more Efficient Topical Dermatologic Therapy, Arch. Dermat. & Syph. 43: 264, 1941.

5. van Zyp, C.: Microchemical Identification of Sulfanilamide, Pharm. Weekbl. 76: 585, 1938.
6. Churg, J., and Lehr, D.: Determination of Sulfanilamide and Its Derivatives in Blood. Am. J. Clin. Path. Tech. Supp. 6: 22, 1942: Simple Micro-test for Sulfanilamide and its Derivatives in Blood. Am. J. M. Sc. 202: 687, 1941.
7. Werner, A. E. A.: Estimation of Sulfanilamide in Biologic Fluids, Lancet 236: 18, 1939.
8. Morris, Colin J. O.: The Determination of Sulfanilamide and Its Derivatives, Biochem. J. 35: 952, 1941.

THE DETERMINATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM BY SPECIFIC GRAVITY MEASUREMENTS*

R. A. Mortensen, Ph.D., and Margaret Hardinge, A.B.
Loma Linda, Calif.

Specific gravity methods[1-4] suitable for the determination of total protein in serum may be applied to the estimation of the albumin and globulin fractions. In a procedure described by Barbour,[5] serum is mixed with an exactly equal volume of saturated ammonium sulfate under oil and is spun in an angle centrifuge for an hour at high speed. The density difference between the supernatant liquid and a like mixture, in which serum is replaced by an accurately standardized dilute solution of ammonium sulfate, gives the basis for calculating the amount of albumin present. Certain rigorous precautions are necessary. On account of the great difference in the specific gravities of serum and saturated ammonium sulfate, a small error in measuring the volume of either solution makes a large error in the result. The necessary degree of precision is seldom attained in routine work.

In the method here described, prolonged centrifuging is unnecessary and the difficulties involved in the determination of the increase in specific gravity due to protein in the presence of high salt concentration are obviated. After precipitation with ammonium sulfate, the globulin fraction is treated with trichloroacetic acid in order to dilute the salt. It is then redissolved in dilute sodium hydroxide. The difference between the specific gravity of the resulting solution and that of a blank, prepared from an equal volume of the trichloroacetic acid supernatant similarly treated with sodium hydroxide, is a function of the protein content of the solution. Globulin and albumin are calculated from this difference and the specific gravity of the original serum. This procedure permits the specific gravity measurements to be made on dilute solutions and within the range used on serum itself. Only ordinary care is required in order to obtain reliable and reproducible results.

METHOD

Reagents.—Ammonium sulfate solution prepared by adding 346 Gm. of the anhydrous salt to 1000 Gm. of water.

Trichloroacetic acid solution, 5 per cent.

Sodium hydroxide solution, 2.25 per cent.

*From the Department of Biochemistry, School of Medicine, College of Medical Evangelists.

Received for publication, June 7, 1943.

This work was aided by a grant from the Alumni Research Foundation of the College of Medical Evangelists.

Procedure.—One cubic centimeter of serum is pipetted into a 15 c.c. conical, graduated centrifuge tube, and 10 c.c. of the ammonium sulfate solution is gradually added while shaking. It is then corked and centrifuged at 2500 to 3000 revolutions per minute for ten minutes. The supernatant liquid is drawn off to the 1.0 c.c. mark and discarded. Ten cubic centimeters of 5 per cent trichloroacetic acid are added and thoroughly mixed with the precipitated protein, using a fine glass rod to break up any coagulum which may be packed in the bottom of the tube. The mixture is now centrifuged a second time at the same speed for five minutes. One-half cubic centimeter of the supernatant liquid is transferred to a test tube for use as a blank; the remainder is drawn off to 0.5 c.c. One and one-half cubic centimeters of 2.25 per cent sodium hydroxide are added to both tubes and the resulting solutions are thoroughly mixed. The precipitated protein is best stirred up to form a paste before the addition of sodium hydroxide and then stirred again immediately afterwards until the protein is completely dissolved.

The specific gravities of the two solutions are measured by any suitable method, such as the well-known falling drop.[2, 3]

In making a series of determinations, is it unnecessary to prepare a blank for each analysis. In fact, it has been found possible to use the same blank reading over a period of several weeks, provided the reagents are kept in well-stoppered bottles.

Calculations.—The globulin, G, in Gm. per 100 c.c., is calculated from the formula

$$G = 695\ D - \frac{T}{10} \tag{1}$$

where D represents the difference in specific gravity between the blank and the protein solution, and T represents the total protein content of the serum in Gm. per 100 c.c.

If measurements are made with the specific gravity pipette, provided with a scale reading directly in percentage of protein as previously described,[4] formula (1) becomes

$$G = 2\ D - \frac{T}{10} \tag{2}$$

in which D is simply the difference between the readings of the blank and the protein solutions.

Albumin is calculated by subtracting the value of the globulin fraction from the total protein.

Example.—A serum specimen was found by the specific gravity method to have a total protein content of 6.8 Gm. per 100 c.c. When analyzed by this procedure, using the falling drop technique of Barbour and Hamilton,[2] the blank and the protein solutions gave apparent density differences, as read from the alignment chart, of 0.0050 and 0.0091, respectively. Substituting the values in formula (1) gives globulin.

$$G = 695\ (0.0091 - 0.0050) - \frac{6.8}{10} = 2.2 \text{ Gm. per } 100 \text{ c.c.}$$

With the specific gravity pipette[4] calibrated to read directly in total serum protein percentage, the readings of the blank and the protein solutions were

5.80 and 7.25 when reduced to the same temperature. Using formula (2) the globulin content is

$$G = 2\ (7.25 - 5.80) - \frac{6.8}{10} = 2.2 \text{ Gm. per 100 c.c.}$$

Remarks.—In carrying out the foregoing procedure, a centrifuge tube should be used which is known to be accurate at 0.5 and 1.0 c.c.

It is important to add the first 2 c.c. of ammonium sulfate solution to the serum a drop or two at a time with shaking; otherwise, the precipitate may be too voluminous. But with this precaution it will seldom occupy a volume greater than 0.6 c.c. In rare cases it may be necessary to centrifuge for a longer time than ten minutes in order to get a clear supernatant liquid. Turbid solutions may result from failure to tightly cork the centrifuge tube.

For drawing off the supernatant solution after centrifugations, it is convenient to use a tapered glass tube connected to a water-aspirator pump. With suction applied to this tube, the solution may be drawn off rapidly and precisely to the required volume without disturbing the precipitate.

The second centrifugation should not be unnecessarily prolonged, as excessive packing of the protein precipitate retards its solution in sodium hydroxide. At times small gel-like particles which dissolve slowly form upon the addition of alkali. Little or no difficulty from this source is experienced, however, when the mixture is stirred immediately.

Small amounts of the ammonia formed in the reaction of sodium hydroxide with the ammonium salt are unavoidably lost; however, the solution may be allowed to stand for as long as an hour after the addition of alkali without introducing significant error, provided the tube is kept well-corked.

With a little experience a complete serum protein analysis can be made in one-half hour, and four or six determinations can be carried out simultaneously in less than one hour. The method is especially suited to the occasional estimation of serum protein fractions, since the reagents are readily prepared and do not require accurate standardization. With the falling drop method no standard other than that commonly used in measuring the specific gravity of serum is required, while the use of the specific gravity pipette dispenses with the need of a standard solution of any kind.

RELATIONSHIP BETWEEN SPECIFIC GRAVITY AND PROTEIN CONTENT

The specific gravity method for the determination of the total protein content of serum is based upon the investigations of Moore and Van Slyke,[1] Weech, Reeves, and Goettsch,[6] Kagan,[3] and Nugent and Towle,[7] who found that a linear relation exists between specific gravity and amount of protein, having the form

$$\text{Total protein} = K\ (\text{specific gravity} - b)$$

where K is a conversion constant and b represents the specific gravity of serum with protein eliminated. Thus, the specific gravity increment of protein, i.e., the contribution made to the specific gravity by the protein present, is directly proportional to the protein content.

Assuming a similar relationship to exist for protein after having been precipitated and redissolved in sodium hydroxide, it follows that the percentage of

globulin present in the serum in the foregoing procedure may be expressed by the general formula

$$G = \frac{k\ a\ V_f\ D}{(a-b)\ V_s} - \frac{b\ T}{a-b} \qquad (3)$$

in which V_s is the volume of serum used, D is the increase in specific gravity due to the presence of protein, k is a proportionality constant, a is the total volume in cubic centimeters of one-half saturated ammonium sulfate, b is the volume remaining after drawing off the first supernatant solution, V_f is the final volume of the solution after the addition of alkali, and T is the total protein in the original serum in Gm. per 100 c.c.

This equation was tested in an extended series of experiments, using different amounts of pooled human serum analyzed for protein by the Howe[8] technique in conjunction with the macro-Kjeldahl nitrogen method. It was found to hold within rather narrow limits over the range of globulin values likely to be encountered. At the same time, the value of constant, k, was found to be 316.

A comparison of this value for k with the corresponding constant in the formula of Weech and co-workers[6] for total protein in serum, where K is taken as 348, indicates that the apparent specific gravity increment of protein under the conditions in the present procedure is about 10 per cent greater than in the case of pure serum. This difference is probably due to a combination of factors, the most important of which is the retention of a certain amount of trichloroacetic acid by the precipitate. That such a retention occurs was demonstrated by Dulière and Minne.[9] The amount retained was found to be proportional to the weight of protein, however; hence, this factor should not affect the validity of formulas (1-3).

Formula (1) may be obtained by substituting into equation (3) the numerical values designated in the procedure, thus

$$G = \frac{316 \times 11 \times 2\ D}{10} - \frac{T}{10} = 695\ D - \frac{T}{10}$$

Formula (1) reduces to formula (2) when specific gravities are replaced by their percentage protein equivalents as given with the direct-reading pipette.

Small departures from the quantities specified in the procedure are possible if the relationships expressed in equation (3) are taken into account. For example, the final volume of solution may be made 1.5 c.c. instead of 2.0 c.c. by the addition of only 1.0 c.c. of sodium hydroxide solution, provided 1.5 is used as V_f in place of 2.0 in calculating the result. There are practical limits to such variations, however. The addition of too small an amount of alkali, for example, results in failure to completely dissolve the protein precipitate, while an increased amount magnifies the effect of errors in the measurement of specific gravity.

In establishing suitable concentrations and volumes for the solutions involved in this method an experimental study was made of the variations in specific gravity with the composition of different mixtures, both with and without protein present. When 2.25 per cent sodium hydroxide was mixed in different proportions with a solution consisting of 10 volumes of 5 per cent trichloroacetic acid and one volume of one-half saturated ammonium sulfate,

the specific gravity of the resulting solutions varied according to curve ACB shown in Fig. 1. With 1.5 per cent protein present the curve EF was obtained. An amount of protein equivalent to this, for example, appears in the final alkaline solution when a determination is carried out on a one cubic centimeter specimen of serum containing 2.3 per cent globulin and 7.0 per cent total protein. The elevation of any point in EF above the corresponding point in CB represents the specific gravity increment, D, in equations (1-3). This quantity is interpreted as due to and proportional to the protein content of the solution. The line EF also represents the approximate maximum range through which the proportions of the two solutions may be varied in carrying out a determination.

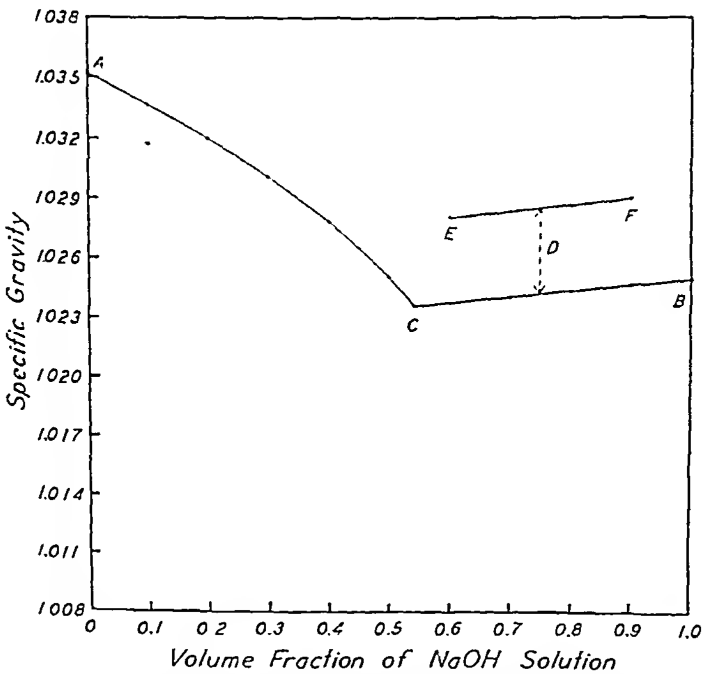


Fig. 1.—Curves representing specific gravity of mixtures of 2.25 per cent NaOH and a solution prepared by adding 10 volumes of 5 per cent trichloroacetic acid to one volume of half saturated ammonium sulfate. Curve ACB, solution containing no protein; curve EF, containing protein. Broken line D represents the increase in specific gravity caused by the presence of protein. Abscissas represent the volume of NaOH solution divided by the sum of the volumes of the two solutions.

RESULTS

Twenty-four determinations by the specific gravity and the Howe-Kjeldahl methods, covering a comparatively wide range of globulin values, are given in Table I. As the method here described is essentially one for the estimation of globulin, values for that fraction only are reported. It will be noted that the correlation between the two methods is close, in fact closer than that usually obtained in a series of total protein determinations. This may be explained in part by the fact that variations in serum constituents, such as salt and glucose, which are soluble in the reagents employed, have no appreciable effect on the result. The standard deviation between the two methods is ±0.14 Gm. per 100 c.c.

SUMMARY

A method for the determination of serum globulin and albumin by specific gravity measurements is described.

TABLE I

COMPARISON OF HOWE-KJELDAHL AND SPECIFIC GRAVITY METHODS FOR SERUM GLOBULIN
Values in Gm. per 100 c.c.

SPECIMEN	HOWE-KJELDAHL	SPECIFIC GRAVITY	SPECIMEN	HOWE-KJELDAHL	SPECIFIC GRAVITY
1	1.7	1.7	13	2.2	2.2
2	3.2	3.1	14	5.1	5.1
3	3.8	3.7	15	1.7	2.0
4	2.1	2.2	16	1.8	1.6
5	4.3	4.0	17	3.0	3.1
6	2.4	2.5	18	1.9	2.0
7	4.3	4.3	19	4.1	4.1
8	3.6	3.6	20	4.9	4.8
9	3.7	4.0	21	5.6	5.6
10	3.7	3.8	22	1.5	1.5
11	2.3	2.3	23	2.7	2.5
12	1.5	1.3	24	2.5	2.5

The globulin is determined by precipitation with one-half saturation of ammonium sulfate, the precipitate being subsequently treated with 5 per cent trichloroacetic acid and redissolved in 2.25 per cent sodium hydroxide. The difference between the specific gravity of the resulting solution and that of a blank prepared from the trichloroacetic acid supernatant liquid represents the amount of protein in the solution, and when used in conjunction with the total protein value gives a basis for calculating both the albumin and globulin fractions.

Any specific gravity method suitable for estimating total serum protein may be employed, and no additional standard is required.

A comparison of the results with those of the Howe-Kjeldahl method is given.

REFERENCES

1. Moore, N. S., and Van Slyke, D. D.: The Relationship Between Plasma Specific Gravity, Plasma Protein Content and Edema in Nephritis, J. Clin. Investigation 8: 337, 1930.
2. Barbour, H. G., and Hamilton, W. F.: The Falling Drop Method for Determining Specific Gravity, J. Biol. Chem. 69: 625, 1926.
3. Kagan, B. M.: A Simple Method for the Estimation of Total Protein Content of Plasma and Serum. II. The Estimation of Total Protein Content of Human Plasma and Serum by the Use of the Falling Drop Method, J. Clin. Investigation 17: 373, 1938.
4. Mortensen, R. A.: A Rapid Method for the Determination of Serum Protein, J. LAB. & CLIN. MED. 27: 693, 1942.
5. Barbour, P. H., Jr.: The Application of the Falling-Drop Method for Specific Gravity to the Determination of Serum Albumin, Yale J. Biol. & Med. 14: 107, 1941.
6. Weech, A. A., Reeves, E. B., and Goettsch, E.: The Relationship Between Specific Gravity and Protein Content in Plasma, Serum, and Transudate From Dogs, J. Biol. Chem. 113: 167, 1936.
7. Nugent, R. L., and Towle, S. W.: The Specific Gravity of Synthetic Solutions of Serum Albumin and Serum Globulin, J. Biol. Chem. 104: 395, 1934.
8. Howe, P. E.: The Use of Sodium Sulfate as the Globulin Precipitant in the Determination of Proteins in Blood, J. Biol. Chem. 49: 93, 1921.
9. Dulière, W. L., Minne, R.: Etude Quantitative sur la Retention de l'Acide Trichloracetique par les Proteines du Serum Humain, Compt. rend. Soc. de biol. 125: 1040, 1937.

Erratum

In the article by Drs. Bernardo Sepulveda and Arnold E. Osterberg, entitled "Serum Bilirubin: A Procedure for the Determination of Indirect and Direct Values," in the August, 1943, issue, page 1359, the third line in the third paragraph should read: "bilirubin is removed from the serum by successive extractions with chloroform."

// The Journal of Laboratory and Clinical Medicine

VOL. 28 NOVEMBER, 1943 NO. 14

CLINICAL AND EXPERIMENTAL

ON THE ABSORPTION AND EXCRETION OF METHYLSALICYLATE ADMINISTERED BY INUNCTION*

R. BEUTNER, M.D., PH.D., B. CALESNICK, M.S., E. POWELL, B.A., AND L. BORTIN, B.A., PHILADELPHIA, PA.

PREVIOUS RESEARCH

THE percutaneous absorption of the salicylates, including methylsalicylate, was investigated as early as 1876 by Drasche,[3] who showed that an alcoholic solution of salicylic acid applied to the skin caused an almost immediate appearance of salicylate in the urine. Since then, numerous other investigators[1, 7] have confirmed the rapid percutaneous absorption of salicylic acid. As this drug proved too irritant for inunction, further attention turned to its esters. Several of these were found to be more rapidly absorbed from the skin, such as methyloxymethyl salicylate, glycol salicylate, and particularly the now preferred methylsalicylate (Impens and Sauerland).[6, 8] In some quarters, however, the percutaneous absorption of the methyl ester is still doubted. Goodman and Gilman,[4] for example, propound the following judgment:

> "Methylsalicylate is . . . absorbed when applied cutaneously, but this route is too uncertain when systemic effects are desired."

There may be some justification for such criticism, as the evidence assembled so far has not been convincing. To wit: Sauerland[8] found that only 0.5 per cent of the applied methylsalicylate was absorbed from the skin, though Impens[6] maintained that the amount absorbed was 8 per cent to 9 per cent; Hanzlik and Preshlo[5] found absorption to be considerably delayed; therefore, they judged it to be slow and irregular; certain other investigations appear unconvincing because of the rather artificial methods of application used, such as the immersion of hands in fatty or alcoholic solutions of methylsalicylate, etc. (Brown and Scott).[2]

*From the Department of Pharmacology, the Hahnemann Medical College and Hospital of Philadelphia.

Received for publication, March 25, 1943.

INVESTIGATION ON A LARGER SCALE

The study here described was done with a larger number of subjects than those reported by previous investigators. A total of 103 inunctions were made on 86 individuals. The series was large enough to lead us to believe that an over-all average may be established in spite of considerable variation from individual to individual, caused by differences in skin permeability, kidney function, etc. The study was undertaken *to determine the salicylate excretion after* inunction of methylsalicylate and to compare it with that occurring after the oral administration of one tablet (0.3 Gm.) of acetylsalicylic acid.

VEHICLES USED

Three different methylsalicylate ointments were used in the study. While their salicylate content was the same (20 per cent), different bases were employed as vehicles:

1. Anhydrous lanolin
2. Anhydrous lanolin and menthol, giving the ointment the following composition:

Lanolin	60 per cent
Menthol	20 per cent
Methylsalicylate	20 per cent

3. A special aqueous base containing:

Glycerin monosterecate	35.0 per cent
Phenolic resin	4.2 per cent
Acacia	3.5 per cent
Water	28.0 per cent
Alcohol	28.0 per cent
Glycerin	1.3 per cent
Total	100 per cent

This rather solid mixture of base 3 was able to emulsify 20 per cent of its weight of methylsalicylate and 20 per cent of menthol, forming a soft ointment which upon inunction was absorbed by the skin without leaving the skin greasy. The lanolin ointments, by contrast, leave a greasy residue, no matter how long they are rubbed on the skin.

As the presented observations will show, the type of ointment used exerted little influence upon the amount of methylsalicylate absorbed. The amount of ointment used for each inunction, regardless of base, was 10 Gm.

TIME LIMITS OF EXCRETIONS

In order to determine the time over which salicylate is excreted, as well after inunction of methylsalicylate as after oral administration of acetylsalicylic acid, urine samples were collected at short intervals after inunction or ingestion, viz., after ¼ hour, ½ hour, 1 hour, and 2, 4, 8, 12, 18, 24 hours. Each sample was tested qualitatively for the presence of salicylate by the addition of a ferric salt; if the urine sample had to be acidified, a small amount of hydrochloric acid was added.

Eight subjects were used for this qualitative determination with methylsalicylate inunctions. In two of these, excretion was limited to two hours; in five, it occurred over a period of 12 hours; only in one case was excretion protracted more than 12 hours.

With acetylsalicylic acid taken by mouth 33 subjects were used. In 31 of these, salicylate excretion was completed in 12 hours; in the other two, a small residual amount was excreted a few hours later.

For the purpose of determining quantitative excretion it seemed justified therefore to limit the time, over which the urine was to be collected to 12 hours.

QUANTITATIVE DETERMINATION

The subjects employed were medical students whose interest in the study assured the proper carrying out of instructions. Each of the subjects was routinely provided with 10 Gm. of a 20 per cent methylsalicylate ointment and instructed to rub the ointment into the skin of his chest, abdomen, and thigh at 6 P.M. without wasting any part of the ointment. If the skin felt uncomfortably greasy after inunction with the lanolin ointments, the excess could be wiped off one-half hour after application, but not sooner.

TABLE I

AMOUNT OF SALICYLIC ACID (IN MG.) EXCRETED AFTER INUNCTION WITH 2 GM. OF METHYLSALICYLATE CONTAINED IN 10 GM. OF AN OINTMENT CONSISTING OF ANHYDROUS LANOLIN 80 PER CENT AND METHYLSALICYLATE 20 PER CENT

NAME	MG. OF SALICYLIC ACID EXCRETED	DEVIATION FROM AVERAGE	VOLUME OF URINE IN 12 HOURS
de Cruz	47.2	5.6	770 c.c.
Corn	50.5	8.9	700 c.c.
Schaffner	50.3	8.7	295 c.c.
Boettger	40.4	1.2	410 c.c.
Darby	19.8	21.8	200 c.c.
Average:	41.6	9.3*	

*Average positive deviation: 7.7.
Average negative deviation: 11.5.

All urine voided from the time of the inunction until the next morning was to be collected in a vessel provided for the purpose. At least two voidings were to be made. The total urine so collected was measured, and 100 c.c. of it were used for determining the salicylate content. Determination was made according to the method of Thoburn and Hanzlik, as follows:

The urine (100 c.c.) and 20 c.c. of added syrupy phosphoric acid were boiled in a 500 c.c. Erlenmeyer flask over a direct flame. The flask was connected with an attachment for conducting steam through it and also with a condenser. Steam was run through the flask, and the distillation was continued until a few drops of the distillate failed to give a pink color reaction when 1 or 2 drops of iron ammonium sulfate were added. When the contents of the Erlenmeyer flask, during the process of distillation, were almost exhausted, or when they foamed and gave rise to fog, water was added and the distillation continued until the distillate proved free from salicylate.

Iron ammonium sulfate was added to the distillate until the violet color of the reaction could no longer be intensified by further addition of the ferric salt. Salicylic acid was then determined colorimetrically by checking against a stand-

TABLE II

AMOUNT OF SALICYLIC ACID EXCRETED (IN MG.) AFTER INUNCTION WITH 2 GM. OF METHYLSALICYLATE CONTAINED IN 10 GM. OF AN OINTMENT CONSISTING OF ANHYDROUS LANOLIN 60 PER CENT, MENTHOL 20 PER CENT, METHYLSALICYLATE 20 PER CENT

NAME	MG. OF SALICYLIC ACID EXCRETED	DEVIATION FROM AVERAGE	VOLUME OF URINE IN 12 HOURS
Gouldin	43.0	12.1	580 c.c.
Juler	92.6	37.5	486 c.c.
Kearney	70.7	15.6	514 c.c.
Graham	61.0	5.9	334 c.c.
Gan	72.5	17.4	448 c.c.
Snyder	74.8	19.7	943 c.c.
Jurewicz	38.8	16.3	416 c.c.
Minnick	49.0	6.1	428 c.c.
Jablonski	90.5	35.4	1155 c.c.
Rigby	40.6	14.5	850 c.c.
Lyons	54.3	0.8	585 c.c.
Chemycz	43.7	11.4	500 c.c.
Neely	43.8	11.3	800 c.c.
Fedullo	16.8	38.3	250 c.c.
Ingersoll	16.5	38.6	1050 c.c.
Gleason	49.0	6.1	486 c.c.
Griffin	78.4	23.3	700 c.c.
Fritz	104.1	49.0	865 c.c.
Ennis	28.6	26.5	340 c.c.
Yamula	34.0	21.1	440 c.c.
Zaydon	43.0	12.1	800 c.c.
Martucci	66.3	11.2	650 c.c.
Average:	55.1	19.5*	

*Average positive deviation: 23.9.
Average negative deviation: 16.6.

TABLE III

AMOUNT OF SALICYLIC ACID (IN MG.) EXCRETED AFTER INUNCTION WITH 2 GM. OF METHYLSALICYLATE CONTAINED IN 10 GM. OF AN OINTMENT CONSISTING OF SPECIAL ABSORBABLE BASE (SEE TEXT) 60 PER CENT, MENTHOL 20 PER CENT, METHYLSALICYLATE 20 PER CENT

NAME	MG. OF SALICYLIC ACID EXCRETED	DEVIATION FROM AVERAGE	VOLUME OF URINE IN 12 HOURS
Munchak	21.0	26.5	350 c.c.
Chepko	94.1	46.6	467 c.c.
Carney	29.1	18.4	715 c.c.
Calvanese	55.2	7.7	400 c.c.
Ballas	54.4	6.9	514 c.c.
Konopka	37.6	9.9	500 c.c.
Wolf	24.5	23.0	368 c.c.
Mozola	27.4	20.1	740 c.c.
Wasley	57.1	9.6	936 c.c.
Witherspoon	46.3	1.2	755 c.c.
O'Brien	30.0	17.5	370 c.c.
Turner	41.9	5.6	935 c.c.
Montalvo-Carroll	55.7	8.2	1105 c.c.
Sussman	97.3	49.8	450 c.c.
Woelfel	41.0	6.5	484 c.c.
Average:	47.5	17.2*	

*Average positive deviation: 21.5.
Average negative deviation: 14.3.

ard solution containing 0.115 Gm. of sodium salicylate per liter; 1 c.c. of this standard solution corresponds to 0.1 mg. salicylic acid; enough iron ammonium sulfate was added to give the maximal pink color (which remains constant for a week).

The quantity of salicylic acid in the 100 c.c. sample was thus determined and, from it, the total quantity of the acid excreted in the urine after inunction with an

ointment containing 2 Gm. of methylsalicylate. The results obtained are listed in the tables shown.

INFLUENCE OF VEHICLES

The vehicle used to present the salicylate to the integument appeared to be of no influence upon the amount excreted in the urine. For confirmation of this finding a number of innunctions with the different bases were made on the same individuals; again the vehicles appeared to be without significant influence (cf. Table IV). When anhydrous lanolin constituted the vehicle, average excretion was 101.3 mg. of salicylic acid; with the absorbable base, 103.1 mg. were excreted; in both instances menthol (20 per cent) had been added to the ointment. Within the limits of experimental error, these figures (amounts excreted) are obviously identical.

It is interesting to note here that the omission of menthol, when the ointment presented methylsalicylate alone in anhydrous lanolin, resulted in a considerably smaller amount of salicylic acid excreted, an average of 64.6 mg. Apparently the addition of menthol promotes greater absorption, hence greater excretion.

On the whole, all averages in this comparative series of innunctions (made on the same individuals) were higher, because a selected group of subjects was used, men who had shown better cutaneous absorption than the average. Dark-complexioned individuals apparently have a higher absorption ability than the blonds.

The data of urinary excretion after oral administration of acetylsalicylic acid are presented in Table V.

CONCLUSION

Tables I to VI show that the following quantities of salicylic acid were excreted:

From Innunction

	Tables I, II, III mg.	Selected Group Table IV mg.
(a) without menthol in lanolin	41.6	64.6
(b) with menthol in lanolin	55.1	101.3
(c) with menthol in absorbable base	47.5	103.1
Average	48.1	89.7

From Oral Ingestion

acetylsalicylic acid, Table V: 65.8 mg.

It is evident that excretion of salicylate after *oral* administration of acetylsalicylic acid (average 65.8 mg.) is on the same level as that from cutaneously applied methylsalicylate (averages 48.1 to 89.7 mg.). If the excretion is virtually the same, it is only logical that absorption into the blood stream and the systemic influence exerted also must have been the same. The conclusion ap-

TABLE IV

EXCRETION OF METHYLSALICYLATE IN URINE AFTER INUNCTION WITH 2 GM. OF THIS SALICYLATE CONTAINED IN 10 GM. OF OINTMENTS WITH THREE DIFFERENT VEHICLES IN EIGHT SELECTED INDIVIDUALS

NAME	LANOLIN	DEVIATION FROM AVERAGE	LANOLIN PLUS 20 PER CENT MENTHOL	DEVIATION FROM AVERAGE	ABSORPTION BASE PLUS 20 PER CENT MENTHOL	DEVIATION FROM AVERAGE
de Cruz	114.0 mg.	49.4	150.4 mg.	49.1	90.6 mg.	12.5
Wolf	140.5 mg.	75.9	99.8 mg.	1.5	127.0 mg.	23.9
Weiss	76.2 mg.	11.6	53.8 mg.	47.5	125.0 mg.	21.9
Rubin	35.8 mg.	28.8	43.7 mg.	57.6	44.8 mg.	58.3
Uzman	82.2 mg.	17.6	98.8 mg.	2.5	200.4 mg.	97.3
Davis	4.1 mg.	60.5	138.0 mg.	36.7	164.3 mg.	61.2
Lapinsohn	35.7 mg.	28.9	48.0 mg.	53.3	21.7 mg.	81.4
Baddour	28.0 mg.	36.6	178.0 mg.	76.7	50.8 mg.	52.3
Average:	64.6 mg.	38.7*	101.3 mg.	40.6**	103.1 mg.	51.1***

*Average positive deviation: 38.6.
Average negative deviation: 38.7.

**Average positive deviation: 54.2.
Average negative deviation: 32.5.

***Average positive deviation: 51.1.
Average negative deviation: 51.1.

TABLE V

AMOUNT OF SALICYLIC ACID (IN MG.) EXCRETED AFTER ORAL INGESTION OF 0.3 GM. OF ACETYSALICYLIC ACID

NAME	MILLIGRAMS OF SALICYLIC ACID EXCRETED	DEVIATION FROM AVERAGE	VOLUME OF URINE IN 12 HOURS
Brzoza	65.8	0.0	940 c.c.
Brobyn	53.7	12.1	350 c.c.
Berio-Suarez	97.6	31.8	390 c.c.
Makel	72.0	6.2	390 c.c.
Lee	53.6	12.2	550 c.c.
Musser	25.2	40.6	760 c.c.
Boyd	133.3	67.5	337 c.c.
Schlossmann	32.2	33.6	350 c.c.
Groth	71.7	5.9	600 c.c.
Gambescia	67.5	1.7	2500 c.c.
Rozanski	45.0	20.8	755 c.c.
Goldstein	114.0	48.2	1850 c.c.
Prunetti	79.7	13.9	475 c.c.
Torres-Oliver	56.3	9.5	700 c.c.
Michaile	128.1	62.3	940 c.c.
Shuttleworth	56.0	9.8	1002 c.c.
Raffa	84.5	18.7	957 c.c.
Souder	29.0	36.8	576 c.c.
Boyer	44.0	21.8	1000 c.c.
Blood	120.4	54.6	507 c.c.
Eckroth	49.1	16.7	636 c.c.
Davis	58.8	7.0	1200 c.c.
Drewery	32.9	32.9	888 c.c.
Guito	50.9	14.9	375 c.c.
Berry	93.7	27.9	422 c.c.
Esgro	48.0	17.8	2000 c.c.
Castagna	60.6	5.2	600 c.c.
Witman	80.2	14.4	750 c.c.
Pigozzi	68.0	2.2	500 c.c.
Kane	37.5	28.3	750 c.c.
Leedom	90.5	24.7	875 c.c.
Flinkman	46.2	19.6	700 c.c.
Galamaga	26.3	39.5	750 c.c.
Average:	65.8	23.0*	

*Average positive deviation: 27.1.
Average negative deviation: 20.0.

TABLE VI

IN A FEW CASES, METHYLSALICYLATE (0.3 GM. IN A CAPSULE) WAS ADMINISTERED ORALLY AND THE EXCRETION STUDIED. AS THE FOLLOWING FIGURES SHOW, THE SALICYLATE EXCRETION WAS APPROXIMATELY AT THE SAME LEVEL AS AFTER ORALLY INGESTED ACETYLSALICYLIC ACID.

NAME	MG. OF SALICYLIC ACID EXCRETED	DEVIATION FROM AVERAGE	VOLUME OF URINE IN 12 HRS.
Mason	128.6	33.0	1180 c.c.
Gambone	42.0	53.6	500 c.c.
Davis	58.8	36.8	1200 c.c.
Thomas	152.4	56.8	1270 c.c.
Average:	95.6	45.1*	

*Average positive deviation: 44.9.
Average negative deviation: 45.2.

Antipyretic Effect of Methylsalicylate Inunction

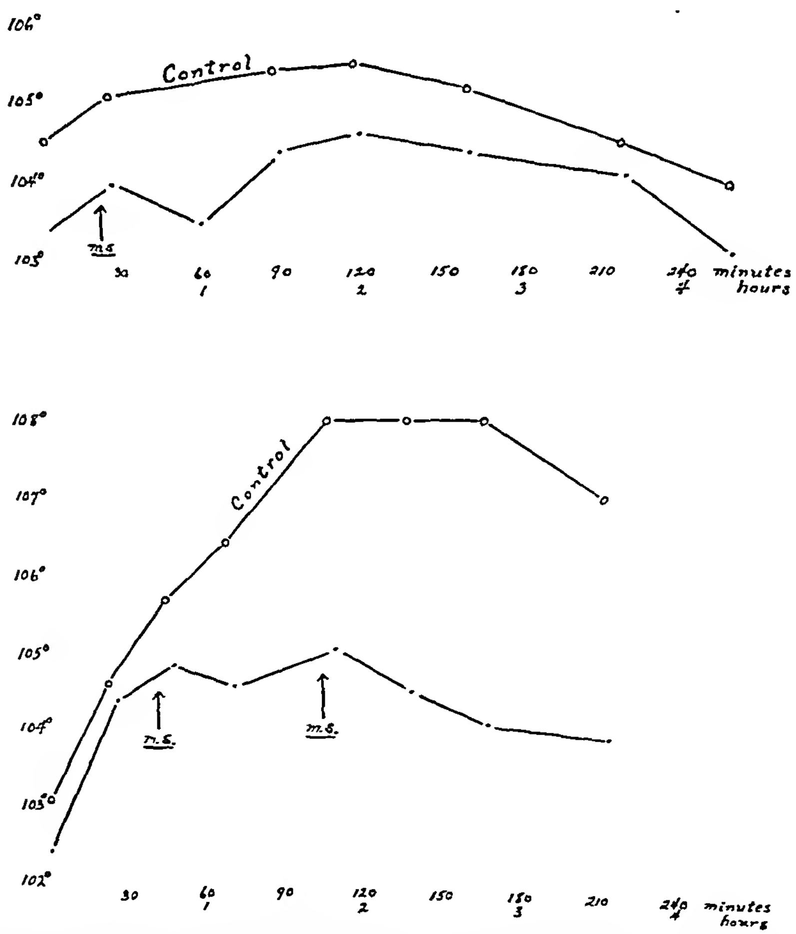


Fig. 1.—In each of these two sets of experiments two rabbits were used; both were injected with 20 mg./kg. of cocaine hydrochloride, whereupon the rectal temperature gradually rose, later slowly returning to normal as indicated. After the application of a 20 per cent methylsalicylate ointment to the ear of one of the animals, however, *m.s.* on graph, a prompt drop of temperature was seen. This drop may be the result of central action of methylsalicylate on the (disturbed) heat center, since we have shown that sizable amounts of this salicylate are absorbed through the skin, or it may be due to a vasodilation resulting from the local irritating effect exerted by the drug. Or both types of action may contribute to the lowering of temperature.

pears justified, therefore, that an adequate inunction of methylsalicylate ointments, of the types described, must be considered internal medication.

ANTIPYRETIC AND VASODILATING EFFECT OF METHYLSALICYLATE

In order to test whether methylsalicylate upon cutaneous application exerts a systemic influence, its antipyretic effect was investigated. Rabbits were injected with cocaine in a quantity of 20 mg. per kilogram of body weight. Within two hours, the temperature of the injected animals gradually rose from approximately 103° F. to approximately 106° F., then slowly returned to normal. When methylsalicylate ointment was rubbed into the ears of the animals, the temperature dropped promptly (see Figs. 1 and 2). This effect may, of course, have been merely the result of the vasodilation produced locally by methylsalicylate, which is a counterirritant.

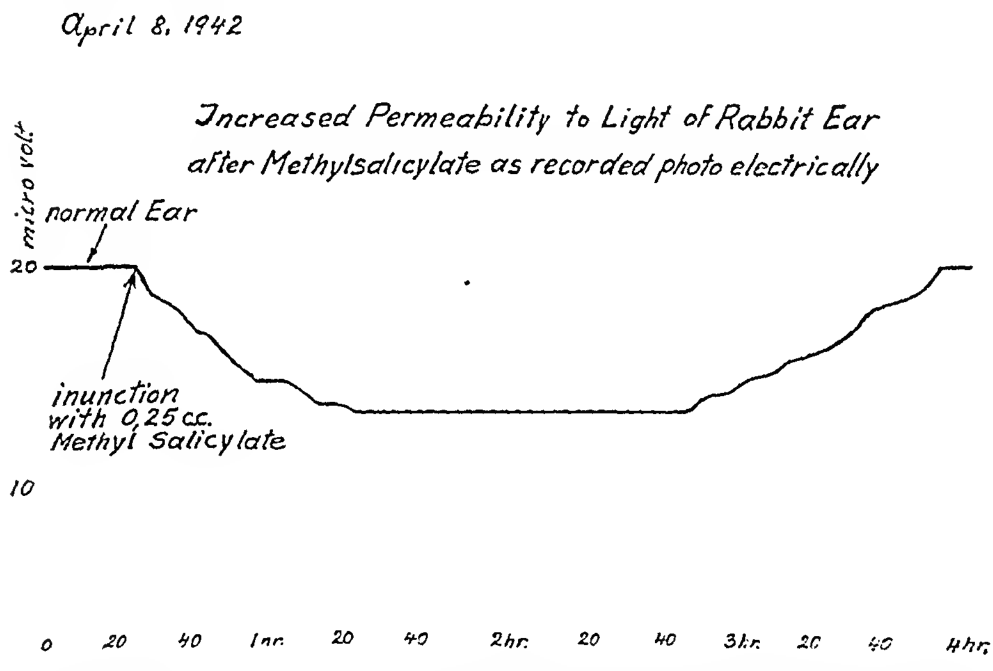


Fig. 2.—The from local application of methylsalicylate on the rabbit's ear was studied ear was held in a frame close to a photoelectric cell (RCA high vacu . The light from a small 3 volt bulb, fed by a 2.5 volt battery was allowed to fall on the rabbit's ear; some of it passed through the translucent tissue and elicited a feeble photoelectric current of the phototube, the voltage of which was recorded continuously on a Leeds and Northrop "Micromax Recorder." The accompanying graph is a twofold magnification of the record thus obtained.

At the point indicated on the graph, the rabbit's ear was rubbed gently with two drops of methylsalicylate, whereupon the photoelectric current decreased due to the greater opaqueness from hyperemia through irritation by the methylsalicylate. Mere mechanical rubbing without a drug had no such effect. (Performed by Mr. George Callé.)

This well-known property of the drug was again demonstrated by the increased ability of a rabbit's ear, after inunction, to transmit light, as measured photoelectrically (see Fig. 2).

SUMMARY

The absorption and excretion of cutaneously applied methylsalicylate was tested in 83 individuals on whom a total of 103 inunctions were made.

Findings were compared with those derived from the oral administration of acetylsalicylic acid.

Similarity of the derived figures (averages) justifies the conclusion that methylsalicylate upon cutaneous application exerts not only its long-established counterirritant influence (vasodilatation, decongestion, induction of active hyperemia), but also a systemic action which perforce must be analgesic, antipyretic, and antirheumatic, since these properties are common to the salicylates upon absorption.

REFERENCES

1. Bourget, Therap. Monatsh. 7: 531, 1893.
2. Brown and Scott, J. of Pharmacol. & Exper. Therap. 50: 32, 1934.
3. Drasche, Wiener med. Zeitschr. 26: 1049, 1876.
4. Goodman and Gilman, Pharmacological Basis of Therapeutics, New York, 1940, Macmillan Co., p. 229.
5. Hanzlik and Preshlo, J. of Pharmacol. & Exper. Therap. 26: 71, 1925.
6. Impens, Arch. f. d. ges. Physiol. 120: 1, 1907.
7. Randolph and Dixon, Med. News 46: 174, 1885.
8. Sauerland, Biochem. Zeitschr. 40: 56, 1912.
9. Thoburn and Hanzlik, J. Biol. Chem. 23: 163, 1915.

SYSTEMIC ALLERGIC REACTION INDUCED BY YELLOW FEVER VACCINE

HARRY SWARTZ, M.D.*

THIS is the record of a case of severe constitutional reaction to the single immunization injection of yellow fever vaccine. It is not the purpose of the writer to detract in the least from the established value of this most efficacious prophylactic agent. In an exhaustive review of the literature, not a single similar case was discovered. When it is considered that upward of several million immunizing injections of yellow fever vaccine have been given to date, the rarity of the case reported below is obvious. It is undoubtedly true that many such reactions of much less severity have been seen and have gone unrecognized or have been recognized and considered of no importance because of their mildness.

Three cases resembling the writer's in minor aspects have been reported by Sulzberger and Asher.[1] These were cases of urticaria and erythema multiforme-like eruptions appearing thirty-six or more hours after yellow fever vaccine injections. Accompanyng the skin manifestations were arthralgias, malaise, fever, pruritus, nausea, and vomiting. As the authors state, the entire symptom complex simulated serum sickness rather than the immediate constitutional reaction of atopy.

It is the intention of the writer to point out by example the necessity of knowing, generally, the constitution of the material injected and of the patient himself.

*Lieutenant, Medical Corps, U.S.A.
Received for publication, March 30, 1943.

REPORT OF CASE

A 27-year-old white male was admitted to the hospital on October 15, 1942, for observation. About one month before admission, he received a single injection of cholera vaccine and one of yellow fever vaccine. Less than five minutes after these injections, while making his way out of the clinic, he became acutely ill. His vision blurred; his breathing became labored and extremely difficult; nausea, vomiting, diarrhea followed and generalized edema of the skin upon which appeared large, raised, pruritic, erythematous wheals. The dyspnea increased in severity, and in a short time he lost consciousness.

After repeated subcutaneous injections of epinephrine, the patient regained consciousness. He was then hospitalized; the severe dyspnea, angioneurotic edema, urticaria, and the gastrointestinal symptoms continued. Epinephrine subcutaneously and ephedrine sulfate orally were maintained in decreasing frequency for the next six days, at which time all symptoms disappeared. He was then discharged from the hospital.

Two days later, while engaged in strenuous physical activity, he experienced a severe paroxysm of dyspnea which lasted two hours. In milder form, it continued for two weeks accompanied by nocturnal wheezy respiration. He was again hospitalized for four days, during which time bronchial symptoms persisted. In addition, a mild generalized urticaria, bullous in character, was noticed. Symptoms subsided and he was transferred to this hospital.

The past history of this patient is of special significance. At the age of four, he contracted the "flux," during which illness he was fed large quantities of egg and egg white. From that time on, he was unable to eat eggs. His inability was initiated not only by a marked aversion for them, but also by the knowledge that ingestion of the smallest fraction of an egg or egg-containing food would precipitate severe asthmatic paroxysms. These paroxysms, he noticed, would also appear on close contact with feathers, e.g., sleeping on a feather pillow or working in the chicken coops on the farm where he lived. In addition to asthma, eggs in any form and in the minutest quantity produced nausea, vomiting, diarrhea, swelling of the face, arms, legs, and generalized itchy wheals. As he grew older, other foods produced the same variety of symptoms in somewhat lesser degree. Those he was definitely able to indict were chicken, pork, chocolate, and peanut. He also noticed that the asthma which had become an almost constant but mild characteristic of his life was most marked during the fall and winter seasons. (His home was in Tennessee.) About six years ago, the attacks decreased in frequency and severity, appearing only at long intervals.

Five years ago, he received a single injection of a vaccine as protection against "flu." A few minutes later, an episode similar to the one described above followed. It was treated with epinephrine and subsided in several hours. The nature of the vaccine could not be determined.

The family history was essentially negative except for his father who had had asthma since childhood.

Physical examination on October 15, 1942, revealed a well-developed, white male, sixty-nine inches tall, weighing one hundred and sixty-five pounds with no significant abnormal physical signs. The blood picture and chemistry, urinalysis, serology showed no abnormalities. The EKG was normal. X-ray of the sinuses showed clouding of the left frontal; others were normal. X-ray of the lungs and chest wall showed no abnormalities.

The allergy investigation, which was as exhaustive as facilities permitted, revealed interesting findings. No precise laboratory investigations were instituted so that the specific modified responsible allergen could not be determined; however, intradermal tests, direct and indirect (passive transfer), and clinical tests were made. All of these pointed incontrovertibly to a single solution of the problem: what was the agent that produced the severe constitutional reaction in this patient? The writer deems it best for clarity to develop these investigations apart from the body of the case report.

The history of this patient, which immediately labelled him an atopic individual with a multivalent sensitivity including a persistent, marked egg

and chicken sensitivity, presented an almost immediate theoretical answer to the severe, near-fatal constitutional reaction following injection of the two vaccines. Yellow fever vaccine was prepared by inoculating eggs incubated seven to eleven days with the 17-D strain of attenuated yellow fever virus developed by Theiler and Smith. This was followed by three more days of incubation, at which time the still-living embryos were aseptically removed from their shells, whatever albumin attached to the embryo not being disturbed. They were ground to a fine pulp in blenders cooled with dry ice, diluted with normal human serum or distilled water, centrifuged for thirty to sixty minutes in an angle centrifuge at about 3,500 revolutions per minute to remove tissue debris. The supernatant extract constituted the vaccine. This was evaporated at a low temperature, standardized, and kept at 4° C. When needed it was dissolved in ten volumes of sterile distilled water and given immediately in 0.5 c.c. doses subcutaneously.[2, 3]

The patient was tested intradermally to more than one hundred allergens. Many inhalant and ingestant skin sensitivities were found. Especially marked reactions were obtained to egg white, chicken meat, and yellow fever vaccine undiluted (four plus). Yellow fever vaccine diluted 1:10 produced a two plus reaction. Cholera vaccine was negative at the end of ten minutes, twenty-four hours, and forty-eight hours. Thus far, proof had been obtained of specific skin sensitivity which confirmed his history of atopy. Because cholera vaccine gave a negative skin reaction both immediate and delayed and because yellow fever vaccine undiluted and diluted 1:10 gave positive skin reactions of the immediate variety, it was established that: (1) cholera vaccine was in no way involved in the constitutional reaction that eventually brought the patient to this hospital, and (2) yellow fever vaccine was undoubtedly the instigating agent.

Clinical tests with the patient's permission both with and without his knowledge (the latter to rule out psychogenesis of resulting symptoms) were performed under close supervision. Both egg and chicken meat were used. Symptoms were reproduced more marked to egg than to chicken meat, mild in degree, controlled with epinephrine.

Clinical sensitivity having been established, it remained only to determine which fraction of the yellow fever vaccine was responsible for initiation of symptoms, the virus fraction or the culture medium fraction. In order to do this, several c.c. of the patient's serum were prepared for passive transfer. The volunteer subject was tested intradermally with the full complement of allergens (more than one hundred), and was found negative to all. Twenty sites on the subject's arm were sensitized, each with one-tenth c.c. of the patient's serum. Forty-eight hours later, these sites were tested with twenty different allergens. At twenty-four-hour intervals, the sites were tested with different series of twenty allergens, until all of those which had produced positive reactions on the patient's skin were tested in this manner. Most of these gave positive reactions on the sensitized sites to lesser degree. Egg transferred four plus; chicken meat two plus; yellow fever vaccine undiluted two plus; yellow fever vaccine 1:10 one plus. All of these reactions, of course, were of the immediate variety. This demonstrated the evident fact that there existed in the serum of the patient (now in the skin of the subject's arm)

reagins for egg, chicken meat, and yellow fever vaccine. Since a virus is not known to be reaginogenic nor is it known to produce skin reactions of the immediate variety, the obvious deduction follows that it was the culture medium fraction of yellow fever vaccine that called forth reaginogenesis.

It was now left to relate this fraction constitutionally and reaginogenically to either egg white, chicken meat, or both. Three widely separated transfer sites were chosen. These were exhausted of egg reagin by successive reinjection with egg extract until no reaction was obtained. These sites were then tested with chicken meat extract. A positive reaction was obtained. When this had disappeared and an hour had been allowed to elapse, these same sites were tested with yellow fever vaccine undiluted. A slight reaction, less than one plus, resulted.

Three different sites were then chosen, again widely separated. These were exhausted of chicken meat reagin in the same manner as above. The sites were then tested with egg extract; a four plus reaction resulted. When the reaction had subsided, an hour was allowed to elapse and the same sites were tested with yellow fever vaccine undiluted. Positive reactions resulted only slightly less marked than the reactions in the sites where neither egg nor chicken meat reagin had been exhausted.

This is conclusive evidence that the reaginic fraction of yellow fever vaccine is related both to egg white and chicken meat structurally and reaginogenically; that it more closely resembles egg white than it does chicken meat. It is known that, from the point of view of both clinical and skin sensitivity, egg white and chicken meat are totally unrelated. In the embryonic development of the chick from the egg, a mutation and differentiation of egg protein take place. It is conceivable that the reaginic fraction of yellow fever vaccine (chick embryo culture) represents a stage in this mutation and therefore by test reveals a kinship to both egg white and chicken meat, albeit the relationship is closer with egg white.

Berger and Hargett,[3] working on the problem of anaphylaxis in guinea pigs following sensitization with chick embryo yellow fever vaccine and normal chick embryo, state, "Chick embryo protein derived from embryos of different ages possesses the power to produce anaphylactic sensitization in young guinea pigs in direct ratio to the age of the embryos employed. . . . ." If one were to transpose this work in the laboratory to the clinical and investigative observations made in this case, the variance would be seen immediately. According to the above authors, the reaginic fraction of yellow fever vaccine should be more closely related to chicken meat than to egg white. This cannot be borne out by the writer's findings.

COMMENT

The presentation of this case of an egg-chicken meat sensitive atopic individual who experienced a severe constitutional reaction immediately following injection of yellow fever vaccine is made to point out the necessity of knowing something of the constitution of the immunizing injection and the subject to whom it is to be given. This emphasizes, by example, the instructions given medical officers of the army relative to questioning the subject about allergic history before administering a vaccine. It is not meant to detract in the least from the justified use of this prophylactic agent.

Evidence is offered in proof of the reaginicity of yellow fever vaccine culture medium and of its close relationship to egg white and chicken meat, more marked to egg white.

It is suggested that marked sensitivity by history or skin test to egg, chicken meat, or both, and/or by skin test to yellow fever vaccine is an indication to give the vaccine carefully, in divided doses with epinephrine at hand in the event a constitutional reaction occurs.

REFERENCES

1. Sulzberger, M. B., and Asher, C.: Urticaria and Erythema Multiforme-Like Eruptions Following Injection of Yellow Fever Vaccine, U. S. Nav. M. Bull. 40: 411, 1942.
2. Kolmer, J. A., and Tuft, L.: Clinical Immunology, Biotherapy and Chemotherapy, Philadelphia, 1942, W. B. Saunders Company, p. 801.
3. Berger, T. O., and Hargett, M. V.: Anaphylaxis in Guinea Pigs Following Sensitization with Chick Embryo Yellow Fever Vaccine and Normal Chick Embryo, Pub. Health Rep. 57: 652, 1942.

THE PHARMACOLOGY OF TWO WATER-SOLUBLE VITAMIN K-LIKE SUBSTANCES*

JAMES J. SMITH, M.S., M.D., A. C. IVY, PH.D., M.D., AND R. H. K. FOSTER, PH.D., M.D., CHICAGO, ILL.

THE object of this study was to test the vitamin K activity, general pharmacology, and toxicity of 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt $6H_2O$ (N-123)† and 2-methyl-1,4-naphthohydroquinone sodium bisulfite complex (MNSS).‡ A preliminary report of this work was presented at the Chicago meeting of The Federation of American Societies for Experimental Biology and Medicine, 1941.[1] Experiments reported here were carried out in the laboratories of Northwestern University and Hoffmann-La Roche and the separate results are designated NU and HLR, respectively. This distinction is made on account of the slightly varying methods and results of the two laboratories.

I. VITAMIN K ACTIVITY

Tests were made on hypoprothrombinemic rats by (a) "Petrolagar" diet;[2] (b) Bile duct obstruction after a preliminary depletion with a diet of low vitamin K content.[3, 4] After depletion, the prothrombin deficient rats were treated with subcutaneous injections of N-123 and MNSS.

METHODS

Production of Deficiency (NU).—Petrolagar diet.—Ninety stock rats weighing from 110 to 230 Gm. were placed on a diet consisting of 70 per cent

*From the Departments of Physiology, Northwestern University Medical School, Chicago, and the Scientific Department, Hoffmann-La Roche, Inc., Nutley, N. J.

Received for publication, March 30, 1943.

†Synkayvite, Hoffmann-La Roche, Inc.

‡Hykinone, Abbott Laboratories, Inc. This substance is an addition product rather than the -3-sodium sulfonate originally reported.[11]

powdered Purina Dog Checkers and 30 per cent Petrolagar. Significant hypoprothrombinemia* developed in 25 per cent of surviving rats in 8 to 96 days as shown by the Quick prothrombin time method.[6] Thirty-two of these rats died in the course of an epidemic of infectious middle ear disease, which swept the colony 30 days after beginning the diet. Thirty-five additional rats were then placed on the diet for a shorter period; of these approximately 50 per cent showed a prolongation of prothrombin time to double or more the original value within 38 days. *Biliary obstruction:* Forty rats were placed on a diet low in vitamin K[5] for periods ranging from 32 to 44 days. In 32 of these the common bile duct was doubly ligated and cut under ether anesthesia. Of 23 rats surviving repeated cardiac puncture and biliary obstruction, 9 or 39 per cent showed a marked hypoprothrombinemia 3 to 10 days following ligation of the common bile duct. During the dietary period there were no significant changes in prothrombin times.

Production† of Deficiency (HLR).—Oil and Oil-Agar Diets.—Petrolagar and later Afkol (a similar preparation) were used, mixing 30 per cent in powdered Purina Dog Chow. Also, 20 per cent mineral oil in the Purina food was used since it kept better and did not mold. Since only a few rats developed significant hypoprothrombinemia, the diet method was abandoned. *Biliary obstruction:* The bile duct was doubly ligated and sectioned. Rats previously on the oil or oil-agar and Purina diet for two months or longer developed prothrombin deficiency in 8 or 10 days. Rats placed on the diet only after operation required 2 to 3 weeks to develop the deficiency. All animals were continued on the diet postoperatively. Seventy-two rats were successfully operated. Of these, 28 died within 2 or 3 weeks though none within the first day or so. Thirty-one deficient rats died following cardiac puncture.

Prothrombin Determination.—Under ether anesthesia 0.45 c.c. of blood was obtained by cardiac puncture (27 gage needle) and drawn into 0.05 c.c. of sodium oxalate solution (1.34 per cent); to this was added 0.5 c.c. physiologic saline, and the specimen was centrifuged. The coagulation time of the plasma was determined by a modified Quick method.[6] To 0.1 c.c. of oxalated plasma at 38° C., 0.1 c.c. of thromboplastin‡ (Quick[7]) was added and quickly thereafter 0.1 c.c. of calcium chloride solution (0.27 per cent). Clotting time was noted with a stopwatch and at least two determinations were run on each sample.

Method of Treatment (NU).—When the prothrombin time had increased significantly (at least twice original value) the animals were injected subcutaneously for two days or more with 2 and 4 gamma doses of both compounds. Prothrombin time (PT) was determined daily. Control animals were injected with physiologic saline, and both substances were dissolved in saline and injected in volumes of 0.5 or 1.0 c.c.

Method of Treatment (HLR).—Depleted animals were injected with single doses ranging from ¼ gamma to 2 gamma per rat. Prothrombin times were determined 18 to 20 hours later. Since the HLR results are reported in terms of per cent prothrombin, a curve relating prothrombin time to per cent pro-

*Prothrombin time increased to twice or more the original level.

†American Pharmaceutical Co., Inc.

‡The dried rabbit brain was placed in glass ampoules sealed under vacuum and appeared to keep indefinitely.

thrombin was determined by plotting data obtained from tests on serial dilutions of plasmas from five normal rats, following the method of Quick.[6]

RESULTS

Results (NU).—Controls.—The mean control reading of prothrombin time in 144 normal rats (both on dietary rats and bile duct obstructed rats) was 22.9 seconds ±0.16 second (S. E. of mean) of ±1.9 seconds (S. D.).

Table I shows the daily PT determinations of 13 rats receiving 0.5 or 1 c.c.

TABLE I

DAILY INJECTION OF SALINE

RAT* NO.	PT** BEFORE DIET	DAYS ON DIET	DAYS POST-OP.	PT BEFORE INJ.	VOLUME INJ. CC.	PT 24 HRS. AFTER 1ST INJ.	PT 24 HRS. AFTER 2ND INJ.	PT 24 HRS. AFTER 3RD INJ.	PT 24 HRS. AFTER 4TH INJ.
P_1	20.7	61		48.6	0.5	47.9	43.9	37.3	
P_2	18.4	28		41.4	0.5	42.0	54.0	43.1	
P_3	22.4	38		41.6	0.5	28.6	28.8	28.6	
P_4	17.8	42		53.2	0.5	47.7	37.4		
P_5	20.2	42		40.7	0.5	51.3	44.8		
P_2	18.4	45		42.0	1.0	52.6	47.3	55.4	63.9
P_6	23.6	34		59.6	1.0	67.8 (Died)			
P_7	21.8	45		44.6	0.5	39.2	35.7	40.2	
P_8	20.6	42		39.8	0.5	39.3	38.5	52.0	
P_9	19.0	92		42.9	1.0	33.8	23.4		
P_{10}	24.3	92		49.2	0.5	50.2	37.2	44.6	
B_1	17.3	44	9	48.0	0.5	48.0	90.0	74.2	91.2
B_2	21.6	36	24	38.8	0.5	38.8	41.7 (Died)		
B_3	19.3	43	11	51.6	0.5	51.6	36.8	47.1	42.6
Mean	20.4			45.72		45.62	43.03	46.94	
Standard error mean				±1.67		±2.71	±4.82	±5.06	

*P = Petrolagar rat; B = Bile duct obstructed rat; the diet in this case was a low vitamin K diet, not Petrolagar.

**P = Prothrombin Time in seconds.

injections of physiologic saline. It is seen that the PT shows rather marked variations from day to day, and in three cases, rats B_3, P_2 and P_9, the injection of saline was accompanied by a considerable reduction of the prothrombin time. Such remissions are thought to be fortuitous and perhaps explainable on daily variations in intestinal absorption. Also, small variations in the vitamin K absorbed are likely to produce magnified changes in prothrombin time when the blood prothrombin level is low, because of the steep slope of the prothrombin time/per cent prothrombin curve in this region. Statistical analysis, however, reveals no significant differences between the mean values for successive days in this control series.

Treated.—In Table II are listed the responses to daily subcutaneous injections of 4 gamma of N-123. The critical ratio ("Student's" "t") between PT before injection and 24 hours after the first injection is 5.03 and between PT before injection and 24 hours after the second injection is 6.32, thus being in both instances highly significant ($P < 0.0002$).

In Table II are shown the responses to subcutaneous injections of 4 gamma MNSS. The critical ratio of the mean PT before injection and after injection is very high (over 6). The critical ratio between the mean value after the first injection and the mean after the second injection is 1.50 (or $P = 0.15$), so most

of the effect of vitamin K was obtained in the first 24 hours. Also in Table II is shown the response of the PT to 2 gamma doses of N-123 and MNSS, respectively.

Storage of Vitamin K in the Body (NU).—In the course of our study, 15 PT determinations were made on 12 rats at various times *after* the deficiency had already been corrected with a series of injections. The results are shown in Table III.

The results show that when the prothrombin time is brought nearly to normal, at 5 days after the last injection it is practically back to the deficient level. These animals received doses of the vitamin K preparations totaling 8 gamma in most cases.

TABLE II

SUMMARY OF RESULTS (NU) ON COMPARATIVE THERAPEUTIC EFFECT OF N-123 AND MNSS

NO. OF RATS	PT BEFORE DIET	PT BEFORE INJ.	PT 24 HR. AFTER 1ST INJ.	PT. 24 HR. AFTER 2ND INJ.	PT. 24 HR. AFTER 3RD INJ.	
11P,* 3B* 14 rats	20.4	45.72 ±1.67	45.62 ±2.71	43.03 ±4.82	46.94 ±5.06	NU, Saline Controls
7P, 6B 13 rats	23.7	57.50 ±3.92	32.31 ±3.21	31.32 ±2.73		NU, 4 gamma N-123
8P, 1B 9 rats	22.5	56.73 ±3.93	31.38 ±1.60	28.45 ±1.12		NU, 4 gamma MNSS
5P, 2B 7 rats	19.4	122.58	37.98 ±3.13	33.18 ±2.65	36.2	NU, 2 gamma N-123
3P, 2B 5 rats	21.4	73.40	43.42 ±3.44	36.74 ±3.08	33.64	NU, 2 gamma MNSS

*P = Petrolagar rats; B = Bile duct obstructed rat.

TABLE III

RETURN OF HYPOPROTHROMBINEMIA FOLLOWING CURE

RAT NO.	PT BEFORE DIET	PT BEFORE INJ.	PT 1 DAY AFTER LAST INJ.	DAYS AFTER LAST INJ.	PT
P_{17}	20.9	45.1	23.6	2	26.9
P_{14}	22.2	44.0	26.9	3	23.0
P_{15}	26.3	58.1	36.5	3	24.7
B_4	23.9	53.4	23.8	3	31.1
B_6	22.6	55.5	28.4	5	48.4
B_4	23.9	41.9	20.3	5	34.9
B_1	17.3	45.8	26.4	5	48.0
P_{23}	21.2	44.8	27.3	6	39.1
P_{20}	17.2	41.3	31.5	6	59.1
B_4	23.9	53.4	23.8	7	54.0
B_4	23.9	54.0	29.2	10	46.2
B_4	23.9	41.9	20.3	10	53.4
B_6	22.6	48.4	35.7	11	42.5
P_{24}	17.2	48.2	27.1	20	34.2
Mean	21.9	48.3	27.2		

Results (HLR).—The mean of 39 PT determinations on normal rats was 21.8 seconds ±0.4 second (S.E. of mean). The standard deviation of a single observation was ±2.5 seconds. These values check well with the NU values.

Injections of N-123 and MNSS were made subcutaneously in deficient rats weighing 150 to 200 grams and PT tests made 18 to 20 hours later. The doses ranged from ¼ to 2 gamma per rat. All deficient rats were markedly jaundiced

and their plasmas were yellow. The results in terms of per cent prothrombin are shown in Table IV. With doses of 2 gamma there was a tendency for the PT to return to a level below 21.8 seconds; that is, the *calculated* per cent prothrombin became higher than normal. This phenomenon does not occur in vitamin K-treated normal animals, however.

When the data are graphed (Fig. 1), it may be noted that with higher doses the two curves approach each other, but that in the lower range there is greater divergence. Doses have been estimated from the curves which give specific effects and these are tabulated in Table V. In addition the relative potencies by weight and molecular weight are given in the latter table. The data in this table demonstrate that on a molecular basis N-123 is 41 per cent more potent than MNSS although on a weight basis it is 26 per cent less potent. These data indicate by indirect comparison that MNSS and 2-methyl-1,4-naphthoquinone (MeNQ) have about equal molecular potencies, since Lee et al.,[8] and Almquist and Klose,[10] respectively, reported N-123 to be 48 per cent and 51 per cent more potent on a molecular basis than MeNQ.

TABLE IV

EFFECT OF VARYING DOSES OF N-123 AND MNSS ON VITAMIN K-DEFICIENT RATS

DOSE GAMMA/RAT	N	N-123 PER CENT PROTHROMBIN BEFORE	N-123 PER CENT PROTHROMBIN AFTER	N	MNSS PER CENT PROTHROMBIN BEFORE	MNSS PER CENT PROTHROMBIN AFTER
1/4	5	33	39	6	38	52
1/2	3	35	67	2	41	80
1	5	31	68	5	39	87
2	2	37	>100	3	37	>100

TABLE V

RELATIVE POTENCIES OF N-123 AND MNSS IN CORRECTING VITAMIN K-DEFICIENCY IN RATS

INCREASE IN PROTHROMBIN PER CENT LEVEL	CORRESPONDING DOSES (ESTIMATED FROM FIG. 2) IN GAMMA: N-123	MNSS	POTENCY OF N-123 RELATIVE TO MNSS (= 1): BY WT.	BY MOL. WT.
15	0.33	0.25	0.77	1.46
20	0.39	0.28	0.69	1.33
30	0.55	0.39	0.71	1.36
40	0.85	0.59	0.70	1.33
50	1.34	1.10	0.82	1.58
	Average		0.74	1.41

On the Mechanism of Vitamin K Action.—While it is now accepted that vitamin K acts by stimulating the production of prothrombin in the liver, the possibility suggested itself, especially earlier, that there might be a direct action in the blood. Particularly because of the greater molecular potency of N-123 over all known vitamin K-like substances, it was deemed advisable to perform some tests in vitro.

Deficient rats whose prothrombin times varied from 30 to 43 seconds were used. Oxalated whole blood and oxalated plasma were obtained and incubated at 37° C. for ½ to 6 hours with amounts of N-123 varying from 0.1 gamma to 10 gamma per 0.1 c.c. Then the regular prothrombin determinations were made. There were no significant changes in the prothrombin time with either

plasma or whole blood. This observation was confirmed, using blood from 2 patients with hypoprothrombinemia and jaundice.

DISCUSSION

These results indicate that both water-soluble substances are capable of correcting prothrombin deficiencies in rats at dose levels of 2 to 4 gamma per day. At these dose levels the results in the Northwestern University laboratory indicated no significant difference between the two compounds (Table II). Since the ratio of their molecular weights is 530:276, this would indicate that N-123 is about twice as potent as MNSS on a molecular basis; however, the data from the Hoffmann-La Roche laboratory indicated only 50 per cent greater potency by molecular weight.

Since it has been established[8, 10] that the molecular ratio of potency, N-123:MeNQ is about 1.50, it follows from Table V that the molecular ratio of potency, MNSS:MeNQ is 1.50:1.41 or 1.06. While this indirect comparison is only an approximation, it suggests that MNSS and MeNQ are probably of about equal potency on a molecular weight basis.

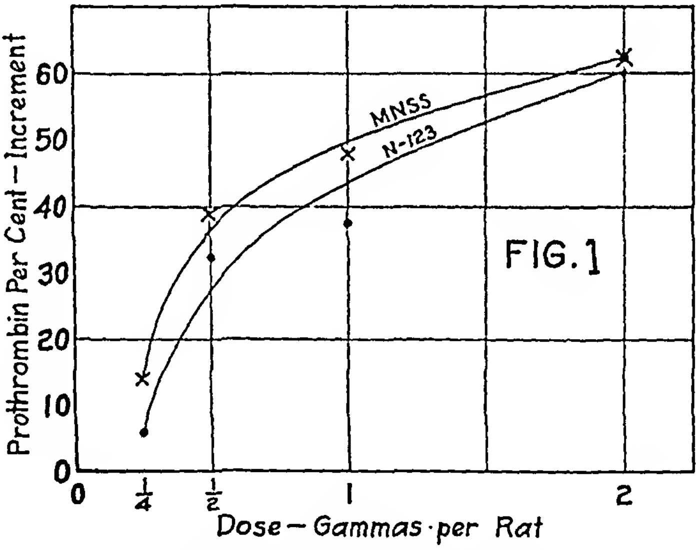


Fig. 1.—Dose-effect curves for N-123 (●) and MNSS (×). Ordinate represents the rise in per cent prothrombin, not the actual per cent prothrombin.

The above ratio, N-123:MeNQ = 1.50, is based on chick assays (both oral feeding and subcutaneous injection). The activity of MeNQ on rats was studied by Flynn and Warner[3] who gave 2 gamma MeNQ daily for two days to bile duct obstructed rats and raised the prothrombin level from 10 to 82 per cent. While the indirect comparisons of parallel results in different laboratories is perhaps equivocal, the Flynn and Warner result is roughly comparable to that of the 2 gamma dose of the two water-soluble substances in Table IV or to the 4 gamma dose in Table II. Taking the latter as the least favorable of the two comparisons, the potency ratio for rats, N-123:MeNQ, is still 1:2 by weight or about 1.54 by molecular weight. This remains to be verified by direct comparison.

II. TOXICOLOGY AND PHARMACOLOGY

Acute Toxicity (NU).—Acute lethal doses were determined by single dorsal subcutaneous injections in albino rats. The animals weighed from 155 to 310 Gm. and there were a few more female than male rats. The results are shown in Table VI. By plotting these data on logarithmic probability paper the LD50's were found to be 610 mg./kg. for N-123 and 175 for MNSS. The ratio, N-123:MNSS, of doses by weight is 3.48, but on a molecular basis (N-123 = 530 and MNSS = 276) the ratio of lethal doses is 1.82.

In a previous communication by one of us[9] the LD50 for N-123 was reported to be 450 mg./kg. in mice by subcutaneous administration and 675 mg./kg. in chicks by the same route.

TABLE VI

TOXICITY BY SUBCUTANEOUS INJECTION IN RATS (NU)

SUBSTANCE	DOSE MG./KG.	N	PER CENT MORTALITY
N-123	450	10	20
N-123	550	10	30
N-123	650	10	60
MNSS	100	10	0
MNSS	150	10	40
MNSS	200	10	60
MNSS	300	5	100

Symptoms appearing in the rat were similar for both drugs and consisted chiefly in a depression of spontaneous activity and some increase in respiration. The depression of activity was noted principally with doses of 550 and 650 mg./kg. for N-123, and with 150, 200 and 300 mg./kg. doses of MNSS. Symptoms appeared 15 to 30 minutes after injection and continued for several hours after which there was a gradual recovery or a progression to death. No convulsions or hyperactivity were seen at any time. Occasionally there was a temporary paresis of the muscles of one forefoot, especially when the injected material extended to the region of the pectoral girdle. These symptoms were of the general order previously described by one of us[9] for other species, except that, particularly in rabbits, the depressive phase was preceded by some movement and agitation and apparent apprehension. These injections were intravenous, however, and in one case (rabbit 324) a convulsion occurred after 30 minutes, the animal dying 5 minutes later. It is of interest that the convulsion occurred during prostration.

With N-123, death occurred in rats in 12 to 70 hours (average = 30 hours), and with MNSS, in 18 to 140 hours (average = 48 hours). The autopsy findings were essentially the same for both drugs. The most constant findings were a bloody nasal discharge, bloody urine, small petechial hemorrhages in the lungs, and moderate enlargement and congestion of the spleen. With MNSS, the heart was frequently stopped in systole, and in these cases there was marked dilatation of both auricles and of the great veins. Microscopic appearances were the same for both drugs, namely, a few cases of marked degeneration of the lining cells of convoluted and collecting tubules of the *kidney* with accumulation of large amounts of protein material in the lumina of the tubules. In

one animal with N-123, the degeneration had progressed to a scattered necrosis of the tubular walls. Glomeruli and blood vessels appeared normal, and there were no significant changes in the liver or adrenals. At the site of injection there was usually moderate congestion of the blood vessels of the subcutaneous tissue but no necrosis.

Chronic Toxicity.—Rats were injected daily subcutaneously with varying doses of the two substances. These data (NU) are given in Table VII. Throughout the injection period, Groups VI, VII, VIII, and IX seemed somewhat more listless than normal. The animals of these groups all showed a loss of weight which was probably significant, especially when contrasted to the Control Group IV which gained in weight. On the contrary, the animals of Groups II and III, receiving only 5 mg./kg. of the two drugs, gained in weight to a greater extent than their control, Group I. While their gain in weight is significant, it is obviously not significantly greater than the gain in the control group, even though the gain of the control group is itself not statistically significant with certainty.

TABLE VII

EFFECT OF CHRONIC ADMINISTRATION ON BODY WEIGHT OF RATS

GROUP	SUBSTANCE	DAILY DOSE MG./KG.	N	NO. OF DOSES	MEAN BODY WEIGHT BEFORE	MEAN BODY WEIGHT AFTER	MEAN BODY WEIGHT PER CENT CHANGE	P
I	Controls	-	10	-	217.9	237.9	+ 9.6	0.085
II	N-123	5	10	20	199.6	226.2	+13.3	0.037
III	MNSS	5	10	20	212.1	239.3	+12.8	0.013
IV	Controls	-	9	-	202.7	214.9	+ 6.0	
V	N-123	25	4	18	221.3	220.7	- 0.3	
VI	N-123	50	4	18	218.5	194.7	-10.9	
VII	N-123	100	4	18	230.0	189.0	-18.8	
VIII	MNSS	25	2	16*	209.0	194.5	- 7.0	
IX	MNSS	50	2	16*	208.0	186.5	-10.3	

*Injections not given on 6th and 7th days because of temporary lack of material.

In Table VIII the data (HLR) from a different series of experiments are given. These animals (mice, rats, and rabbits) were injected for pigmentation studies (see section entitled "Pigmentation"). Injections of N-123 only were made 5 days a week for 4 weeks. The results differed from the preceding in that the animals tolerated much higher daily doses; the 2 days' rest per week from the chemical may explain the difference. None of the mice or rats showed loss of weight up to 300 and 200 mg./kg. daily, respectively. Rabbits developed loss of weight at 200 mg./kg. In this table are also given data on three rabbits injected with methylnaphthoquinone. The loss of weight at a dose of 65 mg./kg. daily was not great, but the question of absorption is present since the material was suspended in polyvinyl alcohol. The dose of 65 mg. is equivalent on a molecular basis to 200 mg. of N-123. In another series of rats injected with N-123 for histopathologic studies all animals without exception gained in weight, the highest dose being 100 mg./kg. for ten doses.

In the series of rabbits injected for hematologic studies there was no significant loss of weight unless the dose was high enough to produce anemia, and even then the results were irregular. In rabbit 357, which received N-123 in 100 mg./kg. daily for 20 doses (5 doses weekly), there was no loss of weight and only a minor slowing up of the rate of growth, even though a moderate

anemia developed. In rabbit 350, which served as a control for 9 weeks and then received four 150 mg./kg. doses, there was a slight loss of weight which developed mostly after cessation of injections, and full recovery from anemia, the loss and recovery paralleling the changes in the red cell count. The anemia in this animal was severe, the red blood cells falling to 1.31 million.

TABLE VIII

PIGMENTATION TESTS WITH N-123 AND MeNQ

Five doses given per week for 4 weeks. Doses were subcutaneous except those starred* which were oral. All but the last three rabbits were treated with N-123.

SPECIES AND NO.	DOSE MG./KG.	INITIAL WEIGHT GRAMS	FINAL WEIGHT GRAMS	PER CENT CHANGE	PIGMENTATION
Mouse 1	100	23	24.7	+ 7	None.
Mouse 2	100	21	23.4	+11	None, died 5 weeks later.
Mouse 3	100	19	21.8	+15	One faint spot on back.
Mouse 4	200	17.5	25.6	+46	None.
Mouse 5	200	17	19.4	+14	None
Mouse 6	200	21.5	25†	+17	Around eyes, back of neck; killed after last injection.
Mouse 7	300	17	21	+24	None.
Mouse 8	300	20	24.7	+24	Scalp and forehead, forelegs, breast slightly spotted; color gone in 5 months.
Mouse 9	300	21.5	23.2†	+ 8	Numerous spots especially on abdomen; accidentally killed after last injection.
Rat 1	100	176	199	+13	Moderate: sides, head, forelegs.
Rat 2	100	169	190	+12	Slight: face, neck, sides.
Rat 3	100	192	205	+ 7	Slight: face, sides, head, perineal region.
Rat 4	200	183	219	+20	Strong: face, sides, sacrum, head, forelegs, perineal region.
Rat 5	200	195	226	+16	Strong: face, neck, abdomen, sacrum, head, all 4 legs.
Rat 6	200	163	195	+20	Strong: head, sacrum, perineal region, trace on sides.
		kg.	kg.		
Rabbit 407	100	2.20	2.62	+19	Slight after 1 week: sides, back and head.
Rabbit 408	100	2.27	2.82	+24	Slight after 1 week: sides.
Rabbit 409	50	2.20	2.67	+21	Slight after 1 week: sides and over eyes.
Rabbit 410	50	2.12	2.90	+37	Slight after 1 week: dorsum, trace on forehead.
Rabbit 368	100*	3.50	3.45†	− 1	None.
Rabbit 369	200*	4.02	3.69†	− 8	None.
Rabbit 412	300*	2.03	1.77	−13	None except margin of lips (contamination?).
The following tests were with MeNQ:					
Rabbit 411	32.5	2.14	2.67	+16	Trace after 1 week over haunches.
Rabbit 370	32.5*	4.20	4.22†	0	None.
Rabbit 371	65*	3.93	3.73†	− 5	None.

†Weight at 3 weeks.

Pigmentation.—The rats in groups VI, VII, VIII, and IX of Table VII developed a peculiar yellowish-brown pigmentation of the hair, which became evident toward the end of the injection period and deepened in the next one to three weeks after the injections were terminated. From then on there was no apparent change in intensity during 10 weeks of observation. The pigmentation, present chiefly in the hair over the face, back, and thorax, was scattered,

but sharply defined, and tended to be symmetrical. This pigmentation has previously been reported by us.[1] This phenomenon was observed in all rats on the higher doses of the two substances in both the Northwestern University and Hoffmann-La Roche laboratories. In addition, rabbits and mice in the latter laboratory were observed to develop the pigmentation (see Table VIII). All rats and nearly all rabbits with sufficient dosage developed it, but mice were less prone to show the color. The color was similar in the three species and always in discrete patches and nearly symmetrical. In all animals the color persisted for months, but finally disappeared. In one rabbit there was only slight fading after six months.

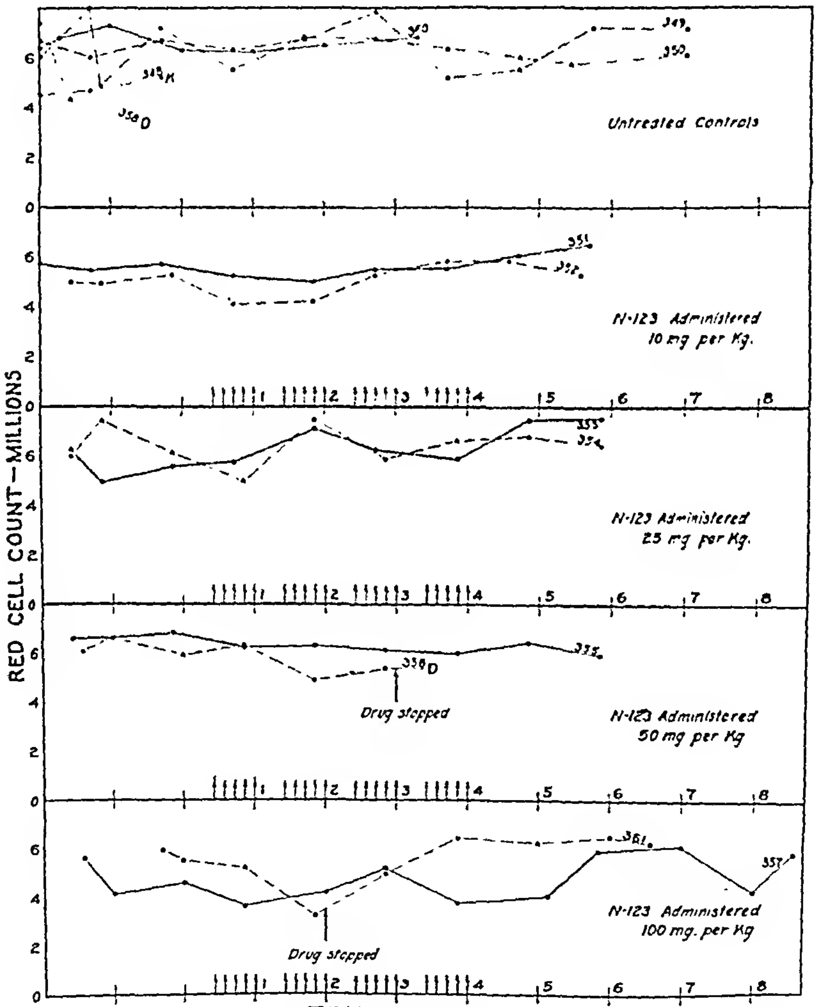


Fig. 2A.—Effect of repeated low doses of N-123 on the red cell count. Arrows indicate the daily injections. D = died.

When N-123 was given orally to rabbits, pigmentation did not occur. Methylnaphthoquinone administered subcutaneously in 3 per cent polyvinyl alcohol solution caused slight pigmentation in one of two rabbits which received 32.5 mg./kg. daily for 20 doses (during 4 weeks). This dose is equivalent to

100 mg./kg. of N-123. At twice the dose administered to one rabbit pigmentation did not occur. There is, of course, the question of absorption here. The MeNQ may possibly have been largely metabolized before absorption. Because MeNQ is irritating there occurred, at the site of nearly every injection, induration which persisted indefinitely, and later a few developed necrosis. The fact that coloring did develop in one rabbit indicates that the phenomenon has its origin in the methylnaphthoquinone nucleus itself, and that the phosphate or sulfonate gives greater effects only because of enhanced solubility. The mechanism of the color production can only be guessed at. A strong solution of N-123 applied daily to the hair had no effect. The color may be due to melanin formation directly or indirectly through metabolic changes in immature hair cells. Possibly vitamins are a factor. The symmetrical and discrete distribution suggests relationship to the color patterns of piebald animals or of nerve and vascular supply.

All rats were colorless after five months. The pigmented rabbits of this series cleared up somewhat faster than previous rabbits in the hematology experiment series.

Micropathology.—Sections from the livers, kidneys, and adrenal glands of the rats in Groups I, II, and III (Table VII) revealed no changes. The doses of N-123 and MNSS were small, 5 mg./kg.

In another experiment on rats (HLR), series of one, five, and ten injections of N-123 were given in 20 and 100 mg./kg. doses. Animals were killed the day after the single dose and three days after the last dose in the other two series. There were no significant changes in the livers or kidneys of any of these animals.

Some of the rabbits under hematologic study were examined. Two rabbits showed no significant gross or microscopic changes after N-123, given subcutaneously in 4 daily doses, 150 mg./kg. One of these animals (No. 349) was killed and autopsied when the red cell count was 1,570,000. The other rabbit (No. 364) was autopsied immediately after death following a convulsion. The convulsion was two days after the last injection, and the red cell count was 1,160,000 on the day of death. Two other rabbits died following similar doses, and delayed autopsy revealed little not attributable to post-mortem changes.

Hematology (HLR).—Eighteen rabbits were started, five serving as controls. Three of the latter were placed in the treated group after several weeks. Coccidiosis appeared in the colony, and one treated (No. 356) and one control animal (No. 358) died. Another control (No. 348) was killed because of severe diarrhea. Aside from these animals, coccidiosis was either mild or did not attack the other animals. N-123 was given daily in doses ranging from 10 to 200 mg./kg. Pronounced effects appeared with doses higher than 100 mg./kg., rabbits receiving the highest doses developing a severe aplastic anemia. Some died, and others recovered following withdrawal of the drug. One animal, No. 350, recovered even though the red blood cells dropped to 1.31 million on the third day after the 4th injection of 150 mg./kg. Following recovery the animals regained normal levels. The red cell counts taken weekly are illustrated in Figs. 2a and 2b. Rabbit 361 showed a drop in red blood cells to 2.90 million after ten 100 mg./kg. doses, but promptly recovered when injections

were stopped. Rabbit 357 actually showed a rise during the first three weeks of injections. Rabbit 360 was injected with 130 mg./kg. a week after the last of four 150 mg./kg. doses. As seen in Fig. 2b there was a temporary relapse after which recovery continued.

Observations were made also on white blood cells and hemoglobin. There were no significant changes except with the highest doses accompanied by severe anemia. In these animals there was usually a polymorphonuclear leucocytosis, but little effect was noted on the eosinophils, basophils, or monocytes. The white cell picture became normal with recovery from the anemia.

Effect on Blood Pressure and Respiration (NU).—Five dogs were used and were anesthetized with sodium pentobarbital. The carotid blood pressure was recorded on a kymograph tracing, and a simultaneous record of respiration was made by means of a pneumograph and tambour. All injections were made intravenously at a rate of 1 c.c. per 3 seconds.

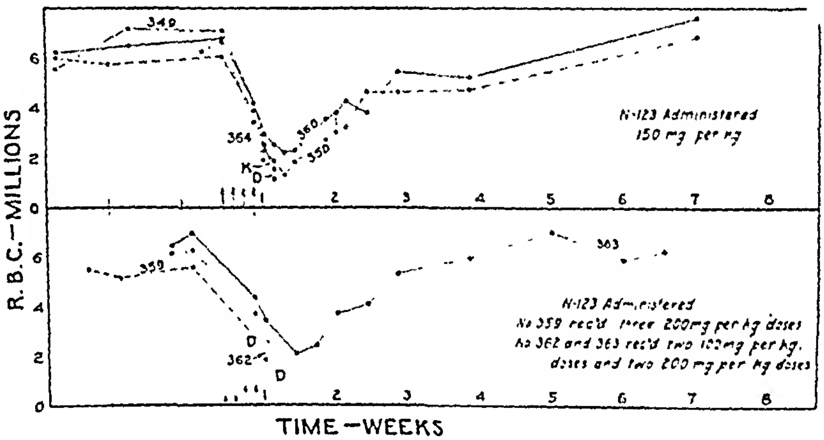


Fig. 2B.—Effect of repeated high doses of N-123 on the red cell count. Arrows indicate daily injections. Half-length arrows indicate a half-size dose in two of the rabbits. Total dose = 600 mg./kg. in all cases, except rabbit 360, which received an additional dose of 130 mg./kg., indicated by the vertical line at second week.

With doses of 20 mg./kg. of either substance, no significant alteration in blood pressure occurred. With 30 mg./kg. of N-123 an elevation in blood pressure began 1 to 2 minutes after the start of the injection, reaching a peak in about 4 minutes and declining gradually to normal in 5 to 10 minutes. No significant secondary increase or decrease occurred. The maximum elevation of arterial pressure was 40 mm. Hg. During this period there was a somewhat decreased respiratory rate. With MNSS 30 mg./kg. resulted in some cases in a transitory slight fall with increased depth of respiration and in other cases no significant changes in either arterial pressure or respiration.

Forty mg./kg. of N-123 caused in one dog a rise in blood pressure of 70 mm. Hg and a greatly increased respiratory rate which was accompanied by a violent stirring about of the animal and an awakening from anesthesia. After 15 seconds, during which time there was no abatement of symptoms, a deeper anesthesia was induced and recovery followed. Similar effects were obtained in cats[9] with 10 mg./kg. doses of N-123 under ether or alurate anesthesia, but the blood pressure reaction was somewhat less under ether. N-123

had little effect on respiration in morphinized rabbits until high doses were given, in which case respiration was depressed.

SUMMARY

1. Two compounds, tetrasodium 2-methyl-1, 4-naphthohydroquinone diphosphoric acid ester $\cdot 6H_2O$ (N-123) and 2-methyl-1, 4-naphthohydroquinone sodium bisulphite complex (MNSS) were studied for vitamin K activity and pharmacologic effects.

2. In bile duct obstructed rats or rats made deficient in prothrombin by dietary means (30 per cent petrolagar in powdered Purina dog chow) 2 to 4 gamma per rat caused a significant shortening of the prothrombin clotting time (Quick method). In one series, 2 gamma of either substance caused the prothrombin time to return to normal and ¼ gamma appeared to be the threshold dose.

3. After the prothrombin time is brought back to normal by the administration of the therapy (about 8 gamma) and then the therapy is stopped, the prothrombin time returns practically to the deficiency level in 5 days.

4. It was shown that vitamin K does not affect prothrombin time on being added to the blood of patients or rats with a prothrombin deficiency.

5. Weight for weight MNSS appeared to be slightly more active, but on a molecular basis N-123 was about 40 per cent more potent.

6. The higher molecular activity of the diphosphate than either MNSS or methylnaphthoquinone is not due to any direct action on the blood in vitro.

7. The LD50 in rats for N-123 is 610 mg./kg. and for MNSS is 175 mg./kg. by subcutaneous injection.

8. In rats receiving high doses of either drug, there was a lowering of spontaneous activity and an increase in respiration.

9. In dogs under pentobarbital anesthesia doses of 30 to 40 mg./kg. caused a marked rise in pressure and respiratory stimulation. MNSS did not exhibit these effects.

10. Lethal doses with both drugs administered to rats resulted in gross and microscopic changes, especially general petechial hemorrhages and renal degeneration.

11. The chronic administration of 100 mg./kg. of N-123 in rats resulted in no pathologic changes.

12. In rabbits, doses of N-123 above 100 mg./kg. caused an "aplastic" anemia from which complete recovery occurred in some animals; others died. Those dying showed no significant microscopic changes directly attributable to the substance. Except for a transient polymorphonuclear leucocytosis during the aplastic anemia there were no significant changes in animals recovering.

13. Mice, rabbits, and rats all developed a yellow to orange-brown pigmentation of the fur in from one to three weeks following a series of medium to large doses of either substance. This pigmentation remained for months with but slow fading. Mice were the least susceptible to the pigmentation phenomenon.

14. The margin of safety between the therapeutically effective and toxic dose of the two preparations is relatively enormous.

REFERENCES

1. Foster, R. H. K., Smith, James J., and Ivy, A. C.: Pharmacological Observations on Two Water-Soluble Vitamin K-Like Substances, Am. J. Physiol. 133: 279, 1941.
2. Elliott, Margaret, Isaacs, Bertha, and Ivy, A. C.: Production of "Prothrombin Deficiency" and the Response to Vitamins A, D, and K, Proc. Soc. Exper. Biol. & Med. 43: 240, 1940.
3. Flynn, Joseph E., and Warner, E. D.: Prothrombin Levels and Synthetic Vitamin K in Obstructive Jaundice in Rats, Proc. Soc. Exper. Biol. & Med. 43: 190, 1940.
4. Greaves, J. D.: Studies on Vitamin K Requirements of the Rat, Am. J. Physiol. 125: 429, 1939.
5. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J.: A New Salt Mixture for Use in Experimental Diets, J. Nutrition 14: 273, 1937.
6. Quick, A. J.: The Nature of Bleeding in Jaundice, J. A. M. A. 110: 1658, 1938.
7. Quick, A. J.: Thromboplastin Reagent for the Determination of Prothrombin, Science 92: 113, 1940.
8. Lee, J., Solmssen, U. V., Steyermark, A., and Foster, R. H. K.: Antihemorrhagic Activity of Tetrasodium 2-Methyl-1,4-Naphthohydroquinone Diphosphoric Acid Ester and Other Naphthoquinone Derivatives, Proc. Soc. Exper. Biol. & Med. 45: 407, 1940.
9. Foster, R. H. K.: Pharmacological Observations on Tetra-Sodium-2-Methyl-1,4-Naphthohydroquinone Diphosphoric Acid Ester, Proc. Soc. Exper. Biol. & Med. 45: 412, 1940.
10. Almquist, H. J., and Klose, A. A.: Comparative Activities of Certain Antihemorrhagic Compounds, Proc. Soc. Exper. Biol. & Med. 45: 55, 1940.
11. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: A Quantitative Study on Blood Clotting: Prothrombin Fluctuations Under Experimental Conditions, Am. J. Physiol. 114: 667, 1936.
12. Smith, H. P., Warner, E. D., and Brinkhous, K. M.: Prothrombin Deficiency and the Bleeding Tendency in Liver Injury ($CHCl_3$ Intoxication), J. Exper. Med. 66: 801, 1937.
13. Baker, B. R., Davies, T. H., McElroy, L., and Carlson, G. H.: The Antihemorrhagic Activity of Sulfonated Derivatives of 2-Methylnaphthalene, J. Am. Chem. Soc. 64: 1096, 1942.

THE EFFECTS OF VITAMIN B DEPRIVATION ON SPONTANEOUS ACTIVITY OF THE RAT*

A. L. Bloomfield, M.D., and M. L. Tainter, M.D.
San Francisco, Calif.

THE effect of vitamin deficiency on activity is not only of biologic interest, but in time of war, when food supplies are short, it is also a matter of great practical moment. There is a general impression among clinicians that deficiency of the Vitamin B complex results in fatigue and sluggishness both mental and physical; this impression has recently been supported by certain purposeful studies. Williams and his associates in 1940 found that women placed on a diet extremely low in thiamine developed inactivity and apathy in addition to loss of weight and prostration. In a later study (1942), in which the thiamine deprivation was less extreme, they also found "physical inefficiency." There were no quantitative measurements. O'Shea, Elsom, and Higbe (1942), however, detected no deterioration in the speed of hand muscle coordination when subjects were deficient in the B vitamins and no improve-

*From the Departments of Medicine and Pharmacology and Therapeutics, Stanford University School of Medicine.
Received for publication, March 30, 1943.

ment after therapy with Vitamin B. Williams and Mason (1942) produced no noticeable symptoms in people placed for six months on a diet supposed to be deficient in riboflavin. Simonson, Baer, and Enzer (1942) made the interesting observation that excessive intake of the Vitamin B complex did not affect ability to perform five different types of muscular work, but the treated subjects felt a subjective improvement of working capacity. Finally Keys and Henschel (1942) showed that additions of vitamins to the standard ration did not improve the physical performance of men in the army.

From the laboratory standpoint Guerrant and Dutcher (1940) found that young rats (23 to 26 days old) placed on a diet deficient in the B complex but supplemented with riboflavin and a rice polish concentrate, so that the food was presumably deficient only in thiamine, were more active for a period of three to four weeks than controls which received thiamine supplements. As the effects of vitamin depletion became severe, activity fell off but increased again when Vitamin B_1 was added to the diet.

Because of the indefinite, and indeed contradictory, character of some of the results in the above reports, it seemed useful to attempt further measurements of the spontaneous activity of rats on deficient diets. The present paper deals with this problem.

METHODS

There is a huge literature on measurement of the activity of the rat under various conditions. Briefly, for our present purpose, it may be said that female white rats, when placed in suitable "running" or revolving cages, display a variable amount of spontaneous activity. This depends upon many conditions: the stage of the estrus cycle, season, temperature, noise, diet, and other factors. Some rats may not run at all; others cover surprising distances in 24 hours, in some cases as many as twenty to thirty kilometers. Most rats when placed in the revolving cages are sluggish at first and then gradually increase their activity until a more or less constant level of daily running is achieved. All these matters have received detailed attention on the part of previous investigators (Slonaker, 1912; Shirley, 1928).

The type of cage used in our experiments (Slonaker, 1908) is a wire mesh cylinder with a circumference of 1.5 meters, which rotates about an axle with very little friction. Each revolution is recorded by a cyclometer on which the total can be read at any time. Within the revolving cage is a smaller stationary box, with food compartment, in which the animal reposes when not running. A battery of twenty such cages were set up in a large room, which for the most part was quiet and had a fairly uniform temperature. The animals were weighed daily, and during the experiments the quantity of food eaten was measured or controlled.

EXPERIMENTS

Experiment 1.—Twenty young female rats, weighing from 80 to 100 grams, were separated into a group of 10 test animals and 10 litter mate controls. Each rat was placed in one of the running cages and was allowed to eat as much of the stock diet* of the laboratory as it desired. The animals and the food were weighed each day and the number of revolutions run was recorded.

*Casein 16, cornstarch 44, yeast 10, salt mixture 4, cod liver oil 10, alfalfa 2 and lard 14.

After a control period of three weeks, when the level of activity seemed stabilized, the ten test rats were changed to a diet deficient in Vitamin B complex. This consisted of washed "vitamin-free" casein 17, cornstarch 64, cod liver oil 10, Crisco 5, and salt mixture 4. On this diet, even though unrestricted in amount, rats soon begin to lose weight; whether because of lack of appetite or because of some specific effect of vitamin deficiency is not certain. The ten control animals continued to receive the stock diet, but the quantity was limited so that they lost weight at about the same rate as the rats on the deficient diet. Thus they served to control any nonspecific effects of inanition. Aside from loss of weight all of the animals appeared well throughout the experiment.

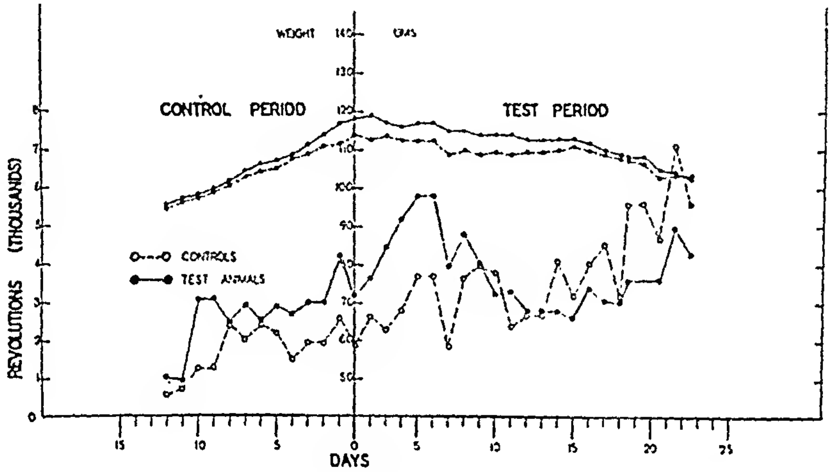


Fig. 1.—Summary of Experiment 1. The first portion of the control period is not shown.

Results of Experiment 1.—The first point to be noted is the great individual variations in activity. These seem important and will be discussed below, but we will present first a composite picture of the test and control animals. Fig. 1 gives the results. The average number of revolutions run daily by the rats in the two groups is shown along with the average weights. The test animals, during the last ten days of the control period, had reached an approximate level of activity in the vicinity of 3000 revolutions. From the start of the test period there is an increase in activity of both sets of rats. This increase, however, is much more marked with the animals on the deficient diet, and within a few days activity is double the control level. The difference in the two groups is statistically significant. After reaching a peak at the fifth day, activity of the test animals falls off rapidly, and within 10 days is back to the control level. A few days later there is again an increase in activity, which coincides with rapid weight loss to a point where the animals' lives are in danger.

In contrast to the test animals, the controls show an entirely different pattern. There is to be sure a slight initial gain in activity, but there is never any drop; from the 15th day on, coincident with rapid weight loss from food restriction, there is a great increase in activity which surpasses that shown by the test animals.

The weight curves require a separate word. During the control period there is the usual brisk gain, almost in a straight line, which occurs with rats of this age on the stock diet of the laboratory. Change to the Vitamin B deficient diet is followed by an immediate slackening in the rate of gain and within a day or two by weight loss, gradual at first, but after approximately two weeks more rapid. The sharp spurt of activity and the subsequent fall are not related to any change in type of the curve of weight loss.

Discussion.—Statistical analysis of the curves of Experiment 1 indicates that the following conclusions may be reliably drawn: (1) Vitamin B deprivation and/or limitation of food intake increase activity initially; (2) limitation of the amount of the normal ration causes progressive rise in activity; (3) vitamin B deficiency after the initial phase of stimulation results in a progressive depression relative to the activity seen in the controls, so that the final (premortal?) stimulation is much less than is to be expected from comparison with the controls in the same state of inanition.

The cause of the increased activity of the rats on the Vitamin B deficient diet is not clear. Guerrant and Dutcher (1940) suggest nervousness from vitamin deprivation as one explanation. However, their young rats which had not reached a steady level of activity before the experiment was started showed increased activity for a period of weeks, an entirely different situation from our experiment in which older rats at a level of activity show only a brief spurt after vitamin deprivation. In other words, a fall in activity in our animals long before serious signs of deficiency are present cannot be well explained on this theory. Another possibility is that change to an inadequate diet which may be distasteful stimulates increased activity in the search for more palatable food.

One can hardly draw any practical applications for man from these experiments, but they suggest that a brief deprivation of Vitamin B may not lessen spontaneous activity, but rather the contrary. It also appears that in rats, at least, restriction of the total amount of food, as in our controls, provided additional vitamins are not fed, tends to increase spontaneous activity slowly over a period of at least three weeks. The final answer to the cause of this phenomenon is not at hand. It is suggestive, however, that in the controls of Experiment 2, to be presented below, where an adequate Vitamin B intake was assured by feeding it to each rat, the same limitation of food did not result in a progressive rise in activity. Hence it is possible that the slow increase in running in the control group in Experiment 1 was the expression of a slowly developing B deficiency brought about by the limitation of food intake and with it of available Vitamin B.

The Results in Individual Rats.—As indicated above, there were striking variations in the activity of individual rats both in the test and in the control series. Among the test animals, for example, No. 6 did not run at all during the entire experiment (see Fig. 2*A*). The cyclometer stood at zero not only during the control period on stock diet but after the Vitamin B deficient diet was instituted. There was no "premortal" increase of activity in spite of very rapid weight loss. The theories discussed above in connection with the composite curve (Fig. 1) all break down as explanations of this animal's

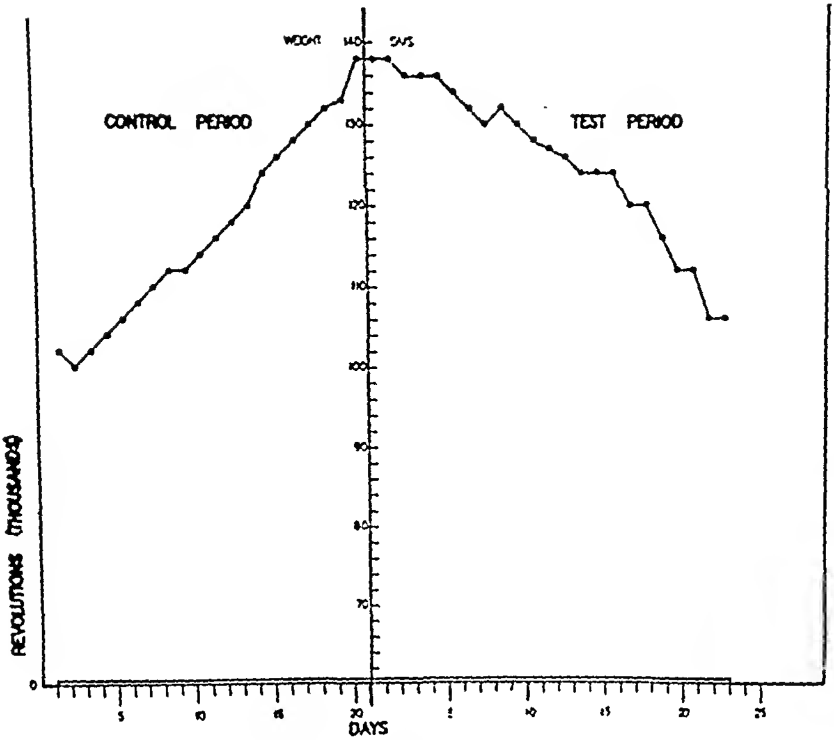


Fig. 2A.

Fig. 2 (*A*, *B*, *C*, *D*, *E*).—Weight curves and activity of individual rats of Experiment 1. See text.

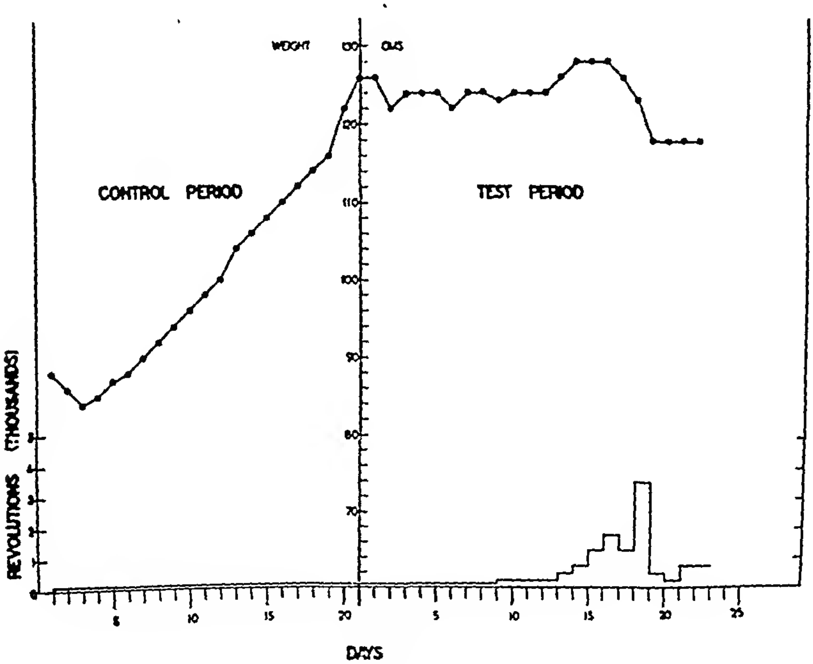


Fig. 2B.

behavior. Rats 5, 4, 2, and 8 of the test series failed to run during the control period and during the early days of the deficiency period, but finally showed slight activity beginning respectively on the seventh, ninth, thirteenth and fifteenth days. There was no correlation, however, between the degree of such activity and the fall in the weight curve (see Figs. 2*B* and 2*C*). Five animals of the test series (Nos. 1, 3, 7, 9, and 10) began to run after the start of the experiment, and by the end of the control period were very active, although the pattern varied in different rats. In No. 7 for example (see Fig. 2*D*) activity was still rapidly increasing at the end of the control period. This rise continued sharply for 6 days; there was then a slight fall with a secondary marked rise even though the animal was not losing weight rapidly at that time. No. 3 (see Fig. 2*E*) failed to show any definite terminal rise in activity even though it was losing weight rapidly.

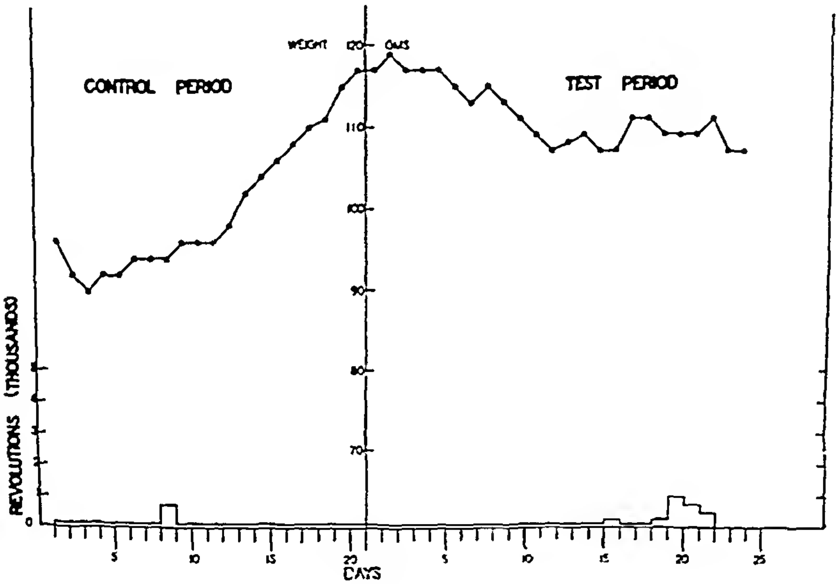


Fig. 2*C*.

Among the control series there were also various types of reaction. One animal failed to run throughout the whole experiment. But whether or not the animals ran during the control period, activity during the test period always increased, even if only slightly, and never showed the invariable spurt of increased activity followed by decline exhibited by the test animals.

Experiment 2.—Because of the possibility that in Experiment 1 the factor responsible for the changes was the alteration in food, rather than the lack of Vitamin B, a second experiment was run in which the food remained unchanged throughout for both groups of rats, and Vitamin B was administered separately. During the control period both test and control animals were placed on the Vitamin B deficient ration described above, but in addition each received 1 c.c. daily of a Vitamin B concentrate which contained according to the manufacturer's statement, in 30 c.c., Vitamin B_1 1500 International Units, riboflavin 1.2 mg., vitamin B_6 4.5 mg., pantothenic acid 12.0 mg., nicotinic acid 60.0 mg. It was also said to contain the filtrate factor (Jukes-Lepkowsky

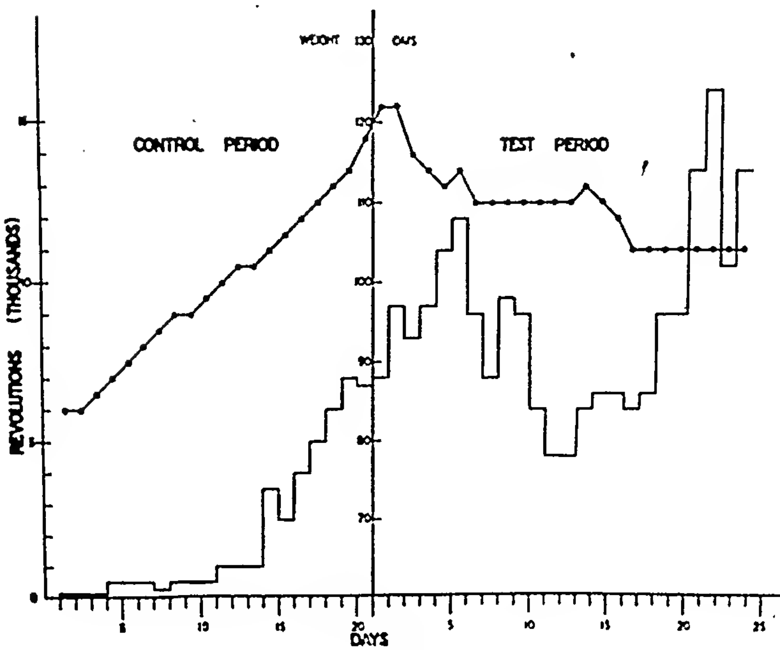

WEIGHT
DAYS
CONTROL PERIOD
TEST PERIOD
REVOLUTIONS (THOUSANDS)
DAYS

Fig. 2D.

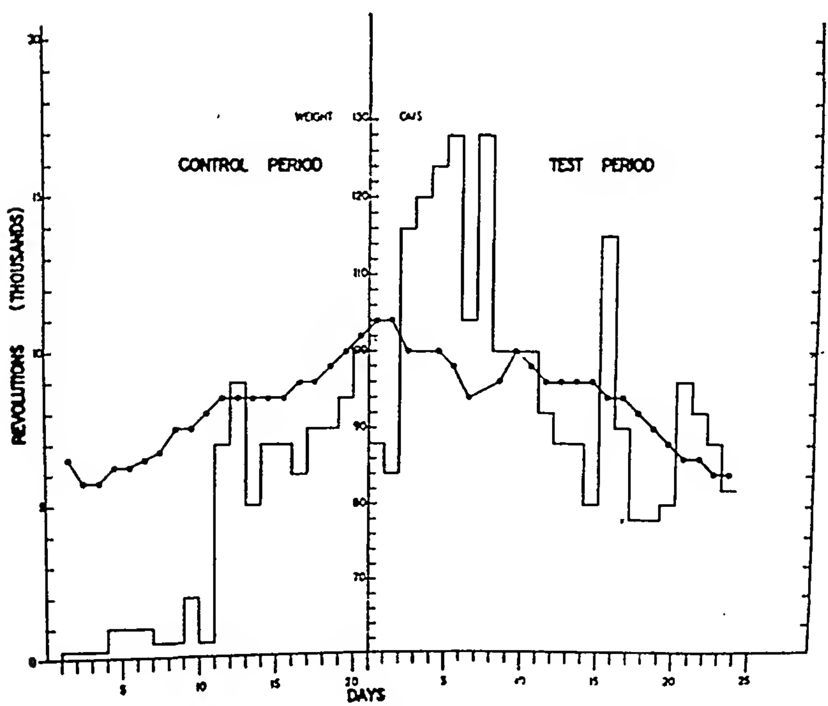

WEIGHT
DAYS
CONTROL PERIOD
TEST PERIOD
REVOLUTIONS (THOUSANDS)
DAYS

Fig. 2E.

value of 28 per c.c.).* This vitamin concentrate was fed separately to each rat, mixed with a few drops of water. At the end of the second control period of 21 days the Vitamin B supplement was withdrawn from the test rats. As these rats lost weight, the quantity of food allowed to the controls was restricted so that they too lost at approximately the same rate, but they continued to receive the full dose of B concentrate. During the control period of 21 days the average gain in weight of the rats in Experiment 2 was 18 Gm. This is definitely less than the gain made by the rats in Experiment 1, which averaged 23 Gm. during the comparable period. We interpret this to mean that the B deficient diet plus B concentrate was not as adequate a ration as the stock diet used in Experiment 1.

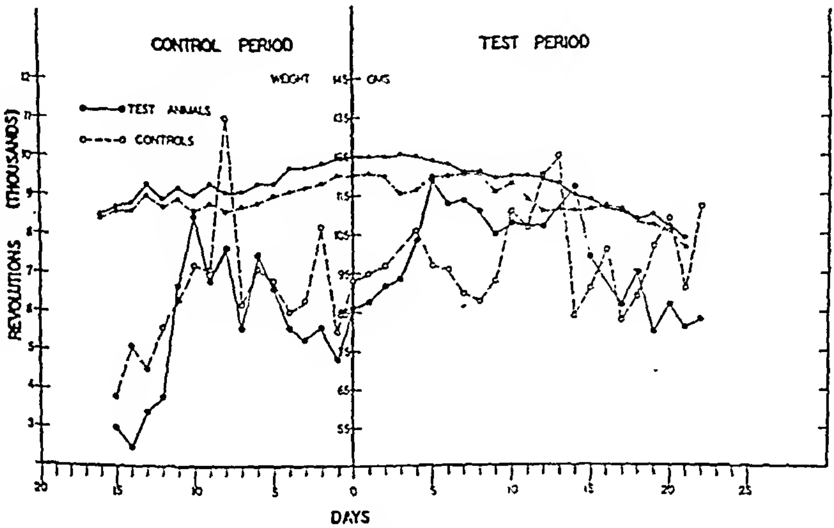


Fig. 3.—Summary of Experiment 2. The first portion of the control period is not shown.

The results (see Fig. 3) are, however, in essential agreement with the first experiment. The animals deprived of the vitamin supplement promptly increased their activity to approximately double the control level; in this case the high level was maintained for a longer period than in Experiment 1. However, there was subsequently a fall to the control level. At this point the experiment was modified by restoring the B concentrate to the test group; the results of this procedure will be discussed later. The control group of rats, although showing considerable irregularity from day to day, did not undergo any consistent deviation from their control level. A statistical analysis of the curves demonstrated that the following conclusions were justified:

1. Vitamin B deprivation was followed by a slightly greater increase of activity than deprivation of food alone. The mathematical chances are 18 to 1 in favor of this being a true difference.

2. After the initial phase of stimulation, Vitamin B deprivation produces a progressive depression of activity as compared to the controls whose food intake is limited without vitamin restriction.

*This was a specially fortified product supplied for this study by the Galen Co., Inc., Berkeley, Calif.

After three weeks both sets of animals were restored to the original diet, that is, they all received the B-free diet as desired plus the 1 c.c. of Vitamin B concentrate just as during the control period. The effect on activity is illustrated in Fig. 4. The six days preceding restoration to the original diet are shown, and it is seen that the activity of the test rats was practically stationary, whereas that of the controls was increasing. On the change to the original diet both sets show an immediate drop in activity to a very low level, which during the ensuing days increases gradually and slightly. The meaning of this interesting finding is not clear. Sudden restoration of vitamins which had been lacking does not suffice as an explanation, because the control group, although restricted in quantity of food, had been receiving the full amount of Vitamin B throughout the experiment. It appears that the correction of either lack of Vitamin B or lack of total calories leads to a decrease of activity, which may be due to release of nervous tension or cessation of need for seeking food. It may simply be that the rats, whose food intake approximately doubled during the first few days of unrestricted diet, were busy eating and therefore ran less, or that the unusual distention of their gastrointestinal tracts caused a disinclination to exercise.

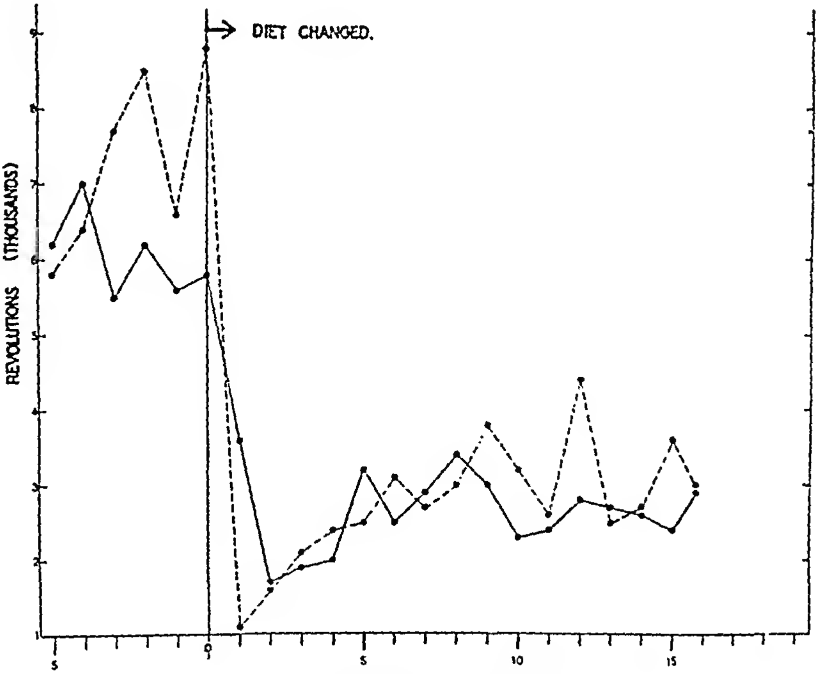


Fig. 4.—Effect on activity of restoration of diet to the conditions obtaining during the control period of Experiment 2.

DISCUSSION AND SUMMARY

To summarize briefly, the object of these experiments was to discover the effect of Vitamin B deficiency on spontaneous activity of the rat. Two experiments along somewhat different lines were done so as to be sure of adequate controls. It was found, in agreement with previous observers, that rats placed in revolving cages do not run at first; they seem to have to learn. In our

observations there was an increase of activity during approximately the first two weeks of the control period; thereafter activity remained more or less on a level. There were great variations, however, in individual animals; some did not run at all during the entire experiment; others became active promptly; some were still increasing their activity at the end of the three weeks' control period.

In any event withdrawal of Vitamin B from the diet was followed by a prompt spurt of activity lasting from five to ten days and then followed by a decline to approximately the control level. This phenomenon occurred with great regularity and was strikingly different from the behavior of the control animals whose food intake was curtailed. These rats showed a slower but continued increase of activity, which went on, with no drop, throughout the course of the experiment, unless a special supplement of Vitamin B was administered.

The explanation of the sharp spurt of activity shortly after vitamin deprivation is not clear. The suggestion of Guerrant and Dutcher that B deficiency causes "nervousness" does not seem adequate to us: first, because the effect is so very prompt, and, second, because there is no evidence that nervousness in the usual sense of the word gives rise to increased running in this type of experiment. During the period of Vitamin B deprivation our rats were very restless, excitable, and difficult to handle. However, this variety of "nervousness" does not necessarily result in increased running, as has been amply demonstrated by one of us (T.) in studies to be reported later. The running response probably requires a conscious purposeful coordinated "drive," which is quite different from the lack of neuromuscular poise or restlessness which is usually called "nervousness." The simplest view would seem to be that shortage of either vitamin or food stimulated the animals into a reaction pattern which would be helpful in the search for a more desirable diet. The drop in activity of the vitamin deficient rats following the initial stimulation seems on the other hand specifically related to Vitamin B lack since it does not occur in the controls. It should be noted that this slowing of activity occurred in our animals long before they showed any visible evidence of deficiency disease; it cannot be regarded at all as an agonal phenomenon.

The sudden drop of activity of rats when either Vitamin B or unlimited calories is restored after a previous period of limitation is also of great interest (see Fig. 4). The only satisfactory explanation is that the fulfillment of a dietary need of any sort is followed by greater food intake, more time spent in feeding, and subsequently in sleeping, and the cessation of the urge to run associated with unsatisfied cravings. This is probably similar to the satiation and tendency to inactivity commonly experienced after an unusually large meal.

Finally, one is tempted to speculate on the bearing of these findings on problems of human nutrition. The suggestion comes forth that in situations where only a small bulk of food can be available, such as in the case of parachute troops or in castaway rations, the supplying of Vitamin B might be just as important over periods of a few days as adequate calories. Our experiments also suggest that Vitamin B deficient diets can cause alterations in

behavior which may precede by many days any other clinical evidence of the vitamin deficiency and may in fact be the only symptom of such deficiency in the subclinical stages.

CONCLUSIONS

1. Limitation of Vitamin B in the diet of the white rat is promptly followed by increase in activity.

2. After a period of a few days to two weeks there is a sharp decrease in activity.

3. This decrease is not to be explained by inanition, since control fasted rats whose Vitamin B intake is maintained at a constant level continue to show normal activity.

4. When rats whose diet has been deficient in either Vitamin B or in total calories are furnished unlimited food and adequate vitamins, there follows, not an increase, but a sudden sharp fall in activity, which persists during the period of increased food intake.

5. Some possible explanations of these phenomena are discussed.

REFERENCES

Guerrant, N. B., and Dutcher, R. A.: The Influence of Exercise on the Growing Rat in the Presence and Absence of Vitamin B_1, J. Nutrition 20: 589, 1940.

Keys, A., and Henschel, A. F.: Vitamin Supplementation of U. S. Army Rations in Relation to Fatigue and the Ability to Do Muscular Work, J. Nutrition 23: 259, 1942.

O'Shea, H. E., Elsom, K. O., and Higbe, R. V.: Studies of The B Vitamins in the Human Subject. IV. Mental Changes in Experimental Deficiency, Am. J. Med. Sc. 203: 388, 1942.

Shirley, M.: Studies of Activity. I. Consistency of the Revolving Drum Method of Measuring the Activity of the Rat, J. Comp. Psych. 8: 1, 1928.

Shirley, M.: Studies of Activity. II. Active Rhythms; Age and Activity; Activity After Rest, J. Comp. Psych., 8: 159, 1928.

Simonson, E., Baer, A., and Enzer, N.: The Influence of Vitamin B (Complex) Surplus on the Capacity for Muscular and Mental Work, Fed. Proc. 1: 81, 1942.

Slonaker, J. R.: The Effect of a Strictly Vegetable Diet on the Spontaneous Activity, the Rate of Growth, and the Longevity of the Albino Rat, Leland Stanford Junior University Publications, University Series, 1912.

Slonaker, J. R.: Description of an Apparatus for Recording the Activity of Small Mammals, Anat. Rec. 2: 116, 1908.

Williams, R. D., Mason, H. L., Wilder, R. M., and Smith, B. F.: Observations on Induced Thiamine (Vitamin B_1) Deficiency in Man, Arch. Int. Med. 66: 785, 1940.

Williams, R. D., Mason, H. L., Smith, B. F., and Wilder, R. M.: Induced Thiamine (Vitamin B_1) Deficiency and the Thiamine Requirement of Man, Arch. Int. Med. 69: 721, 1942.

Williams, R. D., and Mason, H. L.: Observations on Induced Riboflavin Deficiency in Man, Fed. Proc. 1: 192, 1942.

THE REACTION OF LEUCEMIC PATIENTS TO THE SULFONAMIDES

E. L. Amidon, M.D.,* Burlington, Vt.

OUR knowledge of the effectiveness of the sulfonamides on certain infectious diseases is rapidly becoming definite and accurate. Our knowledge of the pharmacologic action of the several forms is still accumulating. The earliest report[1] indicated that the drugs produced a bacteriostasis. The suggestion of the neutralization of the bacterial toxins[2] could only be a partial explanation. Very early it was learned that the temperature greatly affected the activity of the drug. The fact was still left unexplained why certain bacteria were either prevented from multiplying or were killed outright, while other bacteria were seemingly unaffected. Observations of later date suggest the importance played by the medium.[3, 4] It would seem rather certain at the present time that the sulfonamides may destroy certain substances necessary for bacterial multiplication,[5-7] and it is with this theory we are chiefly concerned.

Much experimental evidence has accumulated concerning the toxic effects of the drug. For our purposes only that effect on the white blood cells will be considered. Young[8] apparently was one of the first to report complete agranulocytosis. Many others have demonstrated this change and have offered several explanations. The conclusion of Bliss and Long,[1] that with the exception of toxic changes associated with malignant neutropenia there was no direct effect upon the white cells of the patient, seems adequate. It should be noted that the severe cases of neutropenia usually occur only after the drug has been administered for some time, generally from two to four weeks. Goodman and Gilman[9] suggest that this reaction is dependent upon the factor of personal idiosyncrasy, adding that the reaction is rare. Attention should be called to the fact that when an infection is accompanied by a leucopenia, the administration of the drug may result in a rapid rise in the number of granular white cells.

It remained for Livingston and Moore[10] to demonstrate the effect of sulfapyridine on the white blood cells of patients with leucemia. They administered sulfapyridine to six cases of lymphatic leucemia and reported a definite and marked change in the total number of lymphocytes in the peripheral blood. The first patient with chronic lymphatic leucemia was treated on four separate occasions. The drop in white cells was: from 256,000 to 6,500; from 33,000 to 11,000; from 47,000 to less than 20,000; and from 94,000 to slightly over 20,000. Although this patient was treated late in 1939, the outcome was not stated. Their second patient who apparently developed signs and symptoms of acute lymphatic leucemia showed a drop in the total white count from 212,000 in successive stages of: 183,000 to 100,000 to 60,000 to 40,000 and finally to 20,000. There was no stated evidence of any clinical improvement, and they state that

*Associate Professor and Chairman of Department of Medicine, The College of Medicine of the University of Vermont.
Received for publication, April 9, 1943.

the drug was discontinued on the 19th day because "in spite of the change in blood picture there was clinical evidence of widespread leucemic infiltration."

Following the lead suggested by Livingston and Moore four cases of lymphatic leucemia were studied, and the results tend to agree with those found by the previous investigators.

CASE 1.—Mrs. O. B., 36-year-old, white, was admitted May 4, 1941, complaining of general weakness, edema of the legs, and sore throat of one-week duration. Several small lymph nodes were found in the neck and groin. The spleen was only slightly enlarged. Her blood count showed: hemoglobin, 55 per cent; red cells, 2,520,000; and white count as given below. Several small macular areas and small ecchymotic areas were noted on her legs. In Table I are listed her blood counts with her therapy and progress:

TABLE I

DATE	WHITE CELLS	LYMPHOCYTES	REMARKS
4/ 7	89,000	98	Transfused with 400 c.c. citrated blood.
4/ 9	74,900	98	
4/10			Sulfanilamide started, 1 Gm. every four hours.
4/12	8,350	99	
4/12	5,000	98	
4/13	6,200	95	Sulfanilamide stopped. Transfused 600 c.c. citrated blood.
4/14	13,600	98	
4/15	29,200		
4/16	22,000		
4/17	41,800		Sulfathiazole started 1 Gm. every four hours. Patient irrational.
4/18	20,700		
4/19	5,500	97	
4/21	4,200		Sulfathiazole stopped. Patient very confused.
4/22	2,900		
4/23	1,450		
4/24	2,700	80	Patient irrational.
4/25	2,150		
4/26	2,100		
4/27	1,600	82	
4/28	2,700		
4/29	2,300	69	Condition improved. Temperature normal for first time in hospital.
4/30	3,200		
5/ 1	3,100		
5/ 2	4,200	66	
5/ 5	5,500	36	Neutrophiles, 64 per cent.
5/ 7			Patient up on recliner.
5/ 8	5,400	43	Neutrophiles, 57 per cent.
5/11	7,200	56	Neutrophiles, 44 per cent. Discharged from hospital.

The patient left the hospital with a blood picture of: hemoglobin, 58 per cent; red cells, 2,860,000; and a white count as given above. She received no more of the sulfonamides.

Twenty-seven days after her discharge she was admitted to another hospital. On admission her white count was 32,000 with 97 per cent lymphocytes. On June 14, ten days after admission, with her white count 16,900, she was given sulfathiazole in 1 Gm. doses every four hours. The following day her white count dropped to 3,600, and four days later it was 400, dropping to 300 on the following day. Eight days after sulfathiazole was begun the patient died. Post-mortem examination revealed atrophic bone marrow and splenomegaly. The anatomic diagnosis was acute lymphatic leucemia.

CASE 2.—J. D., a 76-year-old white male, was admitted Sept. 24, 1941, complaining of fatigue, dyspnea, palpitation, and precordial discomfort. His blood count on admission was: hemoglobin, 26 per cent; red cells, 904,000; white count, 306,000; 96 per cent lymphocytes. In Table II are listed his blood counts with his therapy and progress.

TABLE II

DATE	WHITE CELLS	LYMPHOCYTES	REMARKS
9/24	306,000	99	
9/25	576,000	100	Sulfathiazole started 1½ Gm. every four hours for two doses. Pulse weak and thready, respirations rapid and labored, transfused with 750 c.c. blood.
9/26	450,000	99	
9/27			Sulfathiazole started ½ Gm. t.i.d.
9/30	297,600	98	
10/ 2	409,000	99	Patient died.

This patient showed symptoms of vascular collapse only one hour after the second dose of sulfathiazole was given and received only ½ Gm. three times a day after the 27th.

Case 3.—E. P. (H. H.), a 30-year-old white female, for the preceding two weeks had suffered from canker sores in mouth, dysuria, and frequency. A blood count taken during this time showed a hemoglobin of 50 per cent; red cells, 2,400,000; white cells, 6,000 with 24 per cent polys and 76 per cent lymphocytes. She developed abdominal distention and progressive weakness and was admitted to the hospital. Her physical examination on admission showed no enlargement of the spleen or liver, and no enlarged lymph nodes were found. In Table III are listed her blood counts with therapy and progress:

TABLE III

DATE	WHITE CELLS	LYMPHOCYTES	REMARKS
9/29	34,000	90	
10/ 1	46,350	82	Transfused 400 c.c. whole blood.
10/ 3	47,400	90	Sulfathiazole 1 Gm. t.i.d.
10/ 5	37,000	90	
10/ 6	31,000	94	
10/ 7	29,700	93	Patient felt fine, temperature normal for past 24 hours. Sulfathiazole stopped, total of 9 Gm.
10/ 8	23,800	92	
10/10	20,150	86	
10/11	17,550	88	
10/12	18,350	87	
10/14	14,300	72	During past five days has improved, temp. normal.
10/16	42,250	70	Sudden rise in temperature—104° F.
10/17	48,050		Sulfathiazole started, 1 Gm. t.i.d.
10/18			Temperature lower, condition same.
10/20			Patient died.

The patient developed a terminal pneumonia. A post mortem was performed; the diagnosis was acute leucemia.

CASE 4.—H. D. W., a 68-year-old white male, was admitted March 8, 1942, in extremis. About two months previous to admission the patient first noticed a mass in the left axilla which had increased in size. Two weeks previous to admission he noticed several masses in the left side of the neck. These masses also had progressively increased in size. Physical examination revealed enlargement of the liver and spleen. Discrete, nontender, and moderately firm lymph nodes were found in both sides of the neck, axillae, groin, and popliteal spaces. In Table IV are listed his blood counts with his treatment and progress. The temperature which was at 103° dropped progressively after sulfanilamide was given to 100°. The report of the biopsy was acute leucemia, type not determined. On the 18th the patient was well enough to be up and about. His appetite improved as well as his general condition and he was allowed to go home.

The patient was readmitted on May 29, 1942, with a semifluctuant mass on the anterior surface of his right leg, of ten days' duration. He had been well enough to return to his former job as janitor, until ten days previously when he developed "flu." He was referred to the hospital for treatment of the leg. Physical examination revealed large lymph nodes

TABLE IV

TEMP.	DATE	WHITE CELLS	HEMOGLOBIN PER CENT	LYMPHOCYTES PER CENT	RED CELLS	REMARKS
102	3/ 8	201,000	42	99	1,920	Patient very drowsy
	3/ 8	271,000		100		
102.6	3/ 9	303,000	49	99	2,287	
102.8	3/10	213,600	49	99	2,136	Sulfanilamide started, 1 Gm. every 4 hr.
101	3/11	220,000	49	99	2,290	Refuses to eat.
100.6	3/12	170,000	49	99	2,349	Sulfanilamide stopped.
100	3/13	102,400	42	97	1,967	Neck glands dec. markedly.
100.6	3/14	154,600	42	99	2,175	Transfused 500 c.c. whole blood.
98	3/16	204,000	45	97	2,406	Biopsy of node in neck.
100	3/18	240,600	39	98	2,580	Sulfadiazine started, 135 grains in first 24 hours, then 2 Gm. every 4 hr.
99.6	3/20	311,200	49	99	2,928	
99.2	3/23	344,000	49	99	2,486	Sulfadiazine stopped, sulfathiazole started, 135 grains in first 24 hours, total 225 Gm.
98.6	3/25	396,600	39	99	2,306	
98.6	3/27	303,500	45	99	2,077	
98.6	3/28					Discharged.

TABLE V

TEMP.	DATE	WHITE CELLS	HEMOGLOBIN	LYMPHOCYTES	RED CELLS	REMARKS
101	5/30	431,000	32	99	1,519	Patient very drowsy. No appetite.
102	5/31	384,000	36	100		Sulfanilamide started, 1 Gm. every 4 hr.
101.7	6/ 1	300,000		100		
100.4	6/ 2	186,000		99		
100.4	6/ 3	165,000	29	100	1,450	Sulfanilamide stopped.
101	6/ 4	202,000		99		
101	6/ 6	194,000				Sulfanilamide started, 1 Gm. every 4 hr.
100.2	6/ 7	265,000				
102	6/ 8	266,000				
100.4	6/ 9	278,000				
101	6/10	170,000				
100	6/11	192,000				Patient says he feels much stronger.
100	6/12	159,000				Up on recliner.
100.8	6/13	119,000				Sulfanilamido stopped.
101.8	6/15	104,500	32	98	1,655	
100	6/16					Discharged.

throughout body as well as enlarged liver and spleen. The mass on the leg became definitely fluctuant and was incised and drained five days after admission. In Table V are listed his blood counts with his treatment and progress.

The patient ran a septic temperature ranging from 100.4° to 102° for the first four days, after which it ranged between 97° and 101.° The glands in the neck decreased in size during his stay in the hospital.

After the patient's discharge from the hospital, the lymph nodes again enlarged and he grew progressively worse, dying in July of the same year. He did not receive any of the sulfonamides after leaving the hospital.

DISCUSSION

The first patient, suffering from acute lymphatic leucemia, reacted promptly to the administration of sulfanilamide and sulfathiazole by a drop in the total

white cells, followed by clinical improvement from a moribund state to that of comparative health. Contrary to prevailing teachings, the lymphocytes were decreased while the number of neutrophiles remained nearly constant. Because of the prolonged leucopenia, it was thought best to discontinue the sulfonamides.

Patient 2 showed the smallest hematologic change after the administration of sulfathiazole. He received only six Gm. over a four-day period. The sudden vascular collapse after the administration of sulfathiazole is difficult to explain.

The third patient like the first had the acute form of the disease and rallied from a nearly moribund state after sulfathiazole was given. The clinical improvement was marked. It is quite apparent that the drug should have been continued.

The fourth patient, suffering from acute lymphatic leucemia, was in extremis on admission. He responded so well to sulfanilamide that he was able to be discharged from the hospital and was able to return to his job as janitor. On this admission he responded well to sulfathiazole but failed to show any change when sulfadiazine was given.

CONCLUSIONS

It appears that certain of the sulfonamides may produce a rather sudden drop in the circulating lymphocytes in lymphatic leucemia. This does not occur in myelogenousleucemia.[10] That this reaction is not of a toxic nature is best indicated by the suddenness of the reponse and by the fact that there is usually an improvement in the patient's condition.

The suggestion of Stamp[4] and Woods[6] that sulfanilamide may interfere with some "growth factor" inhibiting the development of bacteria is worthy of consideration. Is it possible that the drug acting in a similar manner inhibits the multiplication of lymphocytes? If this is so, it is peculiar that similar results do not occur in myelogenous leucemia, and that sulfadiazine failed to produce similar results.

SUMMARY

Four cases of leucemia are presented, all of which showed a drop in the total circulating lymphocytes, and in all but one, clinical improvement, after the administration of sulfanilamide and sulfathiazole.

I wish to acknowledge with thanks the kindness of Dr. R. E. Miller for the follow-up on Case I, and Dr. W. D. Lindsay in allowing Case 3 to be presented.

REFERENCES

1. Bliss, Eleanor A., and Long, P. H.: Observations on the Mode of Action of Sulfanilamide, J. A. M. A. 109: 1524, 1937.
2. Osgood, E. E.: Culture of Human Marrow, J. A. M. A. 110: 349, 1938.
3. McIntosh, J., and Whitby, L. E. H.: The Mode of Action of Drugs of the Sulfonamide Group, Lancet 1: 431, 1939.
4. Stamp, T. C.: Bacteriostatic Action of Sulphanilamide in Vitro: Influence of Fractions Isolated from Haemolytic Streptococci, Lancet 2: 10, 1939.
5. Green, H. N.: The Mode of Action of Sulphanilamide With Special Reference to a Bacterial Growth-Stimulating Factor ("P" Factor) Obtained From Br. Abortus and Other Bacteria, Brit. J. Exper. Path. 21: 38, 1940.
6. Woods, D. D.: The Relation of p-Aminobenzoic Acid to the Mechanism of the Action of Sulphanilamide, Brit. J. Exper. Path. 21: 74, 1940.

7. Janeway, C. A.: The Sulfonamides. Their Mode of Action and Pharmacology, New England J. M. 227: 989, 1942.
8. Young, C. J.: Agranulocytosis and Para-amino-benzene Sulphonamide, Brit. M. J. 2: 105, 1937.
9. Goodman, L., and Gilman, A.: The Pharmacological Basis of Therapeutics, New York, 1941, MacMillan Co., p. 1039.
10. Livingston, K. E., and Moore, R. D.: The Reaction of Leukemic Patients to Sulfapyridine Administration, New England J. M. 223: 975, 1940.

THE EFFECTS OF VARIOUS INTENSITIES OF LIGHT ON CERTAIN LABORATORY ANIMALS*

MARK E. MAUN, M.D., AND LUVERNE H. DOMEIER, M.D.
DETROIT, MICH.

I. INTRODUCTION

IT IS a common belief that sunlight and well-lighted rooms are healthful. Physicians and lay people assume that sunlight and artificial light are not only beneficial in the treatment and prevention of many diseases but that they promote growth as well. But sunny days and well-illuminated rooms create a desirable environment for most individuals so that the effect of increased light on their well-being is difficult to measure. An obvious lack of control of one's environment makes it yet more difficult to measure the effect of light on the physical, as differentiated from the mental, health of a given group of individuals.

Numerous studies have been made to show the value of ultraviolet therapy, but only a few studies have been made of the effect of visible light on the health and the growth of man and of experimental animals.[6, 24] In our experiment we have attempted to study the influence of various quantities of visible light on the health, the growth, and the behavior of certain laboratory animals.

II. EXPERIMENTAL ROOMS

A. Room Construction.—Three animal rooms were constructed so that no extrinsic light entered them. The rooms measured approximately 16 feet in length, 10 feet in width, and 9 feet in height. Animal cages were placed along one wall of each room, and over them lighting equipment consisting of banks of fluorescent tubes attached to aluminum-covered reflectors was suspended approximately 36 inches above the floors of the cages. The fluorescent tubes were intended to produce approximately 3 foot-candles in one room, 100 foot-candles in the second room, and 1000 foot-candles in the third room. The amount of illumination was measured on the floors of the animal cages. The rooms will be referred to as the 3, the 100, and the 1000 foot-candle rooms, although the levels of illumination did not always reach or remain at the 3, the 100, and the 1000 foot-candle levels (See Chart I).

*From the Department of Pathology, Wayne University College of Medicine, Detroit, Michigan.

Aided by a grant from the Detroit Edison Company.
Received for publication, April 21, 1943.

B. Lighting.—White fluorescent lamps were used in all three rooms to produce the required illumination. The spectral quality of the light is shown in Chart II. Three 15-watt lamps were sufficient to produce 3 foot-candles, while twelve 30-watt lamps produced more than 90 foot-candles in the second room. Sixty-four of the 40-watt lamps plus sixteen of the 30-watt lamps were needed to produce more than 900 foot-candles in the so-called 1000 foot-candle room. The intensity of illumination was measured periodically with a MacBeth illuminometer. Weekly cleaning of the fluorescent tubes to remove dust films was found to prevent a reduction of light output. Some loss of light intensity was noted, however, as the tubes aged, and tube replacements were necessary.

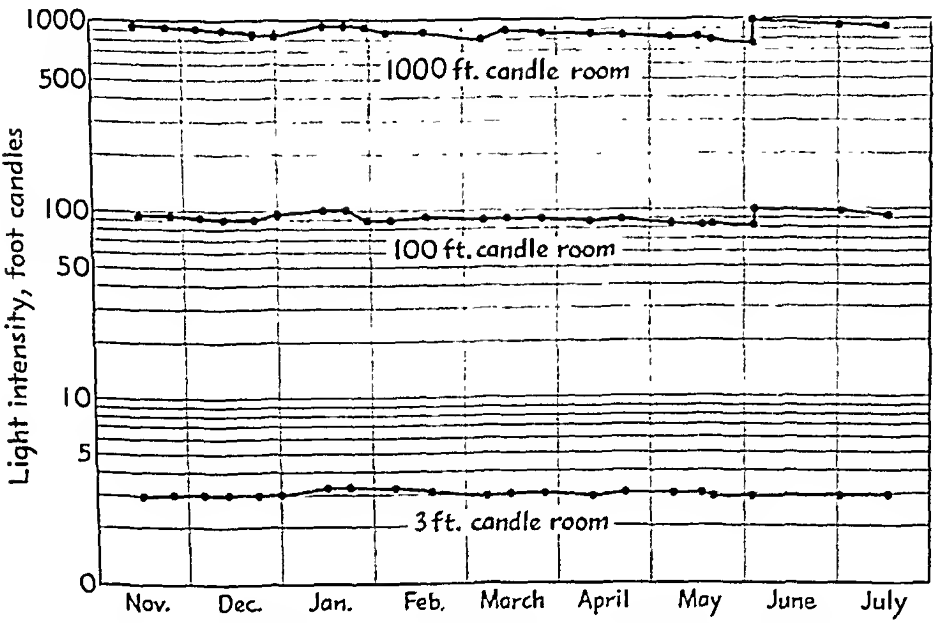


Chart I.—Intensity of light on the floor of the animal cages.

C. Animal Cages.—Animal cages were constructed of thin wire (0.4 mm.) mesh (1 cm. sq.) which permitted a maximum exposure to light; in spite of this precaution, however, there was a loss of approximately 20 per cent as the light passed through the wire mesh. Measurements of light were made inside the animal cages. Glass shields were placed as covers for the cages in the 1000 foot-candle room after removal of the wire tops in an attempt to equalize the radiant energy in the 100: and the 1000 foot-candle rooms. The glass shields were used to absorb and dissipate by convection a considerable portion of the long-wave radiant energy given off by the lamps. The glass covers reduced the radiant energy intensity from 172 British thermal units per square foot per hour to 157 British thermal units per square foot per hour, which figure was considered comparable to 144 British thermal units per square foot per hour found in the 3 and the 100 foot-candle rooms.

D. Temperature and Humidity.—Ventilation of the three rooms was maintained by a 3500 cubic-foot-a-minute fan pulling the warm air of the building into the rooms through light-proof ventilators and exhausting it through a fourth outer room. The air inlets and exhausts were arranged to minimize drafts

on the animals and at the same time to maintain a constant change of air in the rooms. The air velocity in the cages was approximately 15 feet per minute. No attempt was made to control the air temperature or the humidity of the rooms except to avoid extremes. The temperature and the humidity are recorded in Chart III, the peaks seen in the humidity record being due to the washing of the floors and of the fluorescent tubes.

In addition to continuous recordings of the temperature, frequent temperature readings were taken from thermometers placed in the animal cages. In the early days of the experiment the temperature in the 1000 foot-candle room ranged from two to three degrees centigrade higher than in the other rooms, but this elevation subsequently varied less than 1° C. with improved ventilation. These temperature differences were noted after five to six hours of lighting.

E. Time of Exposure.—The animals were exposed to the light in groups varying in size according to the available animal space. The lights were turned on by a time clock at 6 A.M. and turned off at 6 P.M. This period was followed by total darkness. An effort was made to expose the animals from the youngest age at which they could be isolated to young adult life. Although most of the animals were placed in the experimental rooms at 4 to 8 weeks of age, cockerels were placed in the rooms at 48 hours of age. The period of exposure of each animal varied with its health; infection often reduced the duration of the period, for animals were sacrificed if it appeared that infection would be fatal. Healthy animals were sacrificed at the termination of an arbitrarily chosen growth period.

F. Animal Care.—All animals were given superior care and attention. The cages were kept scrupulously clean, and sick animals were promptly placed in isolation cages, where they continued to receive, nevertheless, the same exposure to light. A large Toledo scale was provided for weighing the animals at four-to-five-day intervals.

III. ANIMALS

Guinea pigs, rabbits, rats, and leghorn cockerels were used in the study. These animals were used because of our familiarity with them and because of the ease with which they can be handled. Since some of this group might be considered nocturnal, there is some doubt as to the wisdom of the selection. Many of the guinea pigs and rabbits were albinos, but there were some mixtures of color. The rats were all albinos, and the chickens were all white leghorns.

Animals of both sexes were used, and an attempt was made to obtain litter mates. There was difficulty, however, in obtaining them, and even when they were obtained, the destruction of a litter by loss of one or more animals made the consideration of litter mates of doubtful value in the final analysis. The cockerels were chosen indiscriminately.

The animals received unlimited quantities of food from shielded hoppers which were so constructed as to allow a minimum amount of food to be exposed to the light. The rats were given ground dog chow, while the rabbits and the guinea pigs received rabbit chow. The chickens were given a varied diet consisting of chicken mash, cracked corn, sour milk, oyster shells, and occasionally some ground dog food. Water in unlimited amount was furnished to all animals by means of continuous feed bottles. The water in the cages of the rabbits,

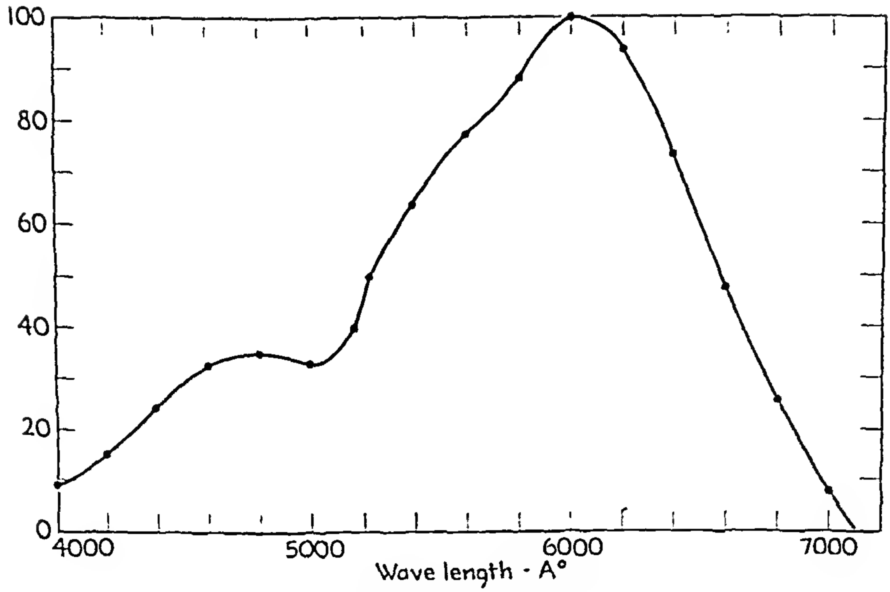


Chart II.—Spectral quality of light in the animal rooms.

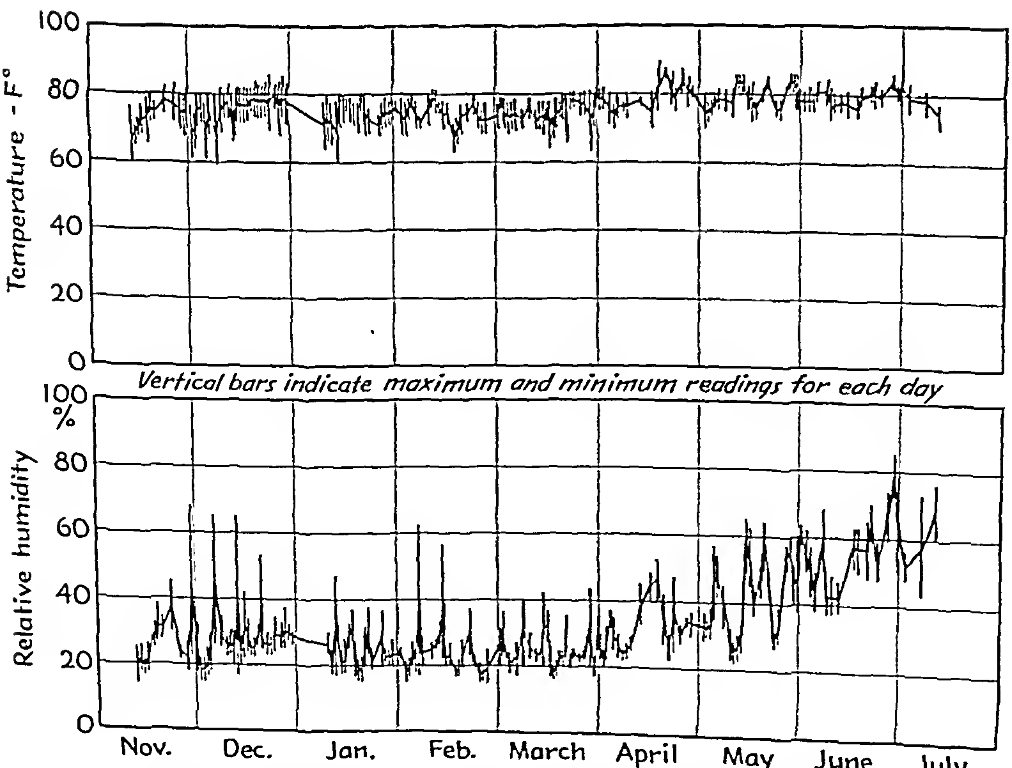


Chart III.—Record of temperature and humidity in the animal rooms.

the guinea pigs, and the chickens was necessarily exposed to light, but the water drunk by the rats was protected in feed bottles. Accurate measurements of the quantity of food and water consumed were not made, but we believe that the amount of food consumed was approximately the same in all rooms. More water was apparently consumed in the 1000 foot-candle room.

IV. RESULTS

A. General Behavior of Animals

1. Activity and Comfort.—The experimental animals were observed closely to determine whether there were any differences in their reactions to exposure to the varying quantities of light. In general there were only slight differences, and these were most evident in comparing the animals in the 1000 foot-candle room with those in the other two rooms. Rats exposed in the 1000 foot-candle room seemed at times to be apathetic, and it was noted that they did not huddle, but tended to lie apart and upon their backs. The rats were most apathetic when the temperature in the 1000 foot-candle room exceeded that of the other rooms, so we attributed some of the differences in activity to the differences in temperature. Rabbits and guinea pigs seemed to be equally active in all rooms. The cockerels in the 1000 foot-candle room had a tendency to pant more readily than the birds in the 3 and the 100 foot-candle rooms. All experimental animals in the 100 foot-candle room were as comfortable and active as animals in the 3 foot-candle room. Apparently the intensity of light does not prove uncomfortable to animals if the temperature is not elevated.

2. Health.—The health of the animals was as good as could be expected in view of the fact that animal quarters were limited. In all rooms some of the rabbits developed numerous attacks of severe diarrhea associated with anorexia and marked loss of weight. These infections proved to be highly contagious so that prompt isolation was necessary. Though a few of the animals succumbed to infection, the majority of them recovered without specific therapy. The considerable weight loss by the rabbits during infection was promptly regained with improved health.

The rats remained in excellent health throughout the experiment, and losses were negligible. Respiratory infections and snuffles were at a minimum while the growth and the gain in weight were constant.

The guinea pigs remained in good health in all experimental rooms during the first ten to fifteen days of the experiment, but after this period they developed shaggy hair, anorexia, diarrhea, muscle weakness, and marked loss of weight, and were sacrificed. We believe that the picture was similar to experimentally produced vitamin C deficiency.

The cockerels were in excellent health throughout the entire experiment. A few of the unfeathered birds succumbed in the early days of the experiment because of low temperature for chicks. Subsequently the chickens were vigorous and very active. Those in the 1000 foot-candle room panted with an elevation of temperature.

3. Sensitivity to Light.—The experiment provided an opportunity for observing the reactions of the animals to high levels of illumination. Our daily observations should be of some practical value, for animals may be more sensitive to light than man. We paid particular attention to behavior, to at-

tempts to hide, and to the appearance of the feathers or of the animals' skin. As far as we could determine, all animals appeared to be unaffected by the high levels of illumination in the 100 and the 1000 foot-candle rooms. No attempts to hide from the light were noted. Examination of the skin of the animals revealed the color, the texture, and the vessels to be comparable in all rooms, and no evidence of abnormal pigmentation was present. The feathers of the chickens were normal to gross examination; the combs of the chickens in all rooms appeared to be paler and larger than normal. This latter change was attributed to the minimal amount of ultraviolet rays present in the light.

	MALE					FEMALE				
	Animal number	Color	Total weight gain (grams)	Growing days	Average daily weight gain (grams)	Animal number	Color	Total weight gain (grams)	Growing days	Average daily weight gain (grams)
3 ft. candle room	1	B	1203	86	13.9	4		187	8	23.3
	2	B	1070	56	19.1	5		993	41	24.2
	3	B	1361	56	24.3	6		1078	41	26.3
						7		594	29	20.4
						8		547	25	21.8
						9	W	3007	86	34.9
						10	W	436	55	7.9
						11	W	218	10	21.8
						12	W	880	38	23.1
	Average		1211.3	66.0	19.1	Average		882.2	37.0	22.63
100 ft. candle room	13		325	9	36.1	14		649	29	22.4
						15		414	14	29.5
						16		1078	40	26.9
						17		498	41	12.1
						18		461	25	18.5
						19	WB	675	47	14.3
						20	WB	2027	87	23.3
						21	W	1616	39	41.4
						22	W	1686	39	43.2
	Average		325.0	9	36.1	Average		1011.5	40.1	25.62
1000 ft. candle room	23	W	2089	89	23.4	25		584	41	14.24
	24	B	1000	58	17.2	26		222	10	22.2
						27		626	25	25.0
						28		623	25	24.9
						29	B	2152	88	24.4
						30	W	518	12	43.1
						31	W	1421	40	35.5
	Average		1544.0	73.5	20.30	Average		892.2	34.4	27.04

Chart IV.—Record of the growth of female rabbits.

4. Growth.—Charts IV through X furnish evidence of the growth of the experimental animals. The rate of growth per day was considered a fair index of the effect of the varying quantities of light upon the animals, and comparison of the growth of all animals in the 3, the 100, and the 1000 foot-candle rooms has been made. Only the animals that survived the experimental period and those living sufficiently long to be significant are compared in the charts.

a. Rabbits.—Examination of Chart IV reveals the growth record of 25 female rabbits, an obviously small number. The growth period of the animals was variable because of the loss of some of the animals by infection. The

average daily weight gain was practically the same; the average gain of the animals in the 1000 foot-candle room, 27.04 grams, was only slightly greater than that of the animals in the 100 and the 3 foot-candle rooms, 25.62 and 22.63 grams respectively. The comparison of litter mates, as suggested before, did not prove to be useful. Only a few male rabbits survived the experimental period so that any study of their relative growth would be of little value. In the course of the experiment it became evident that the maintenance of health in rabbits in small quarters is difficult.

	MALE					FEMALE				
Room	Animal number	Color	Total weight gain (grams)	Growing days	Average daily weight gain (grams)	Animal number	Color	Total weight gain (grams)	Growing days	Average daily weight gain (grams)
3 ft. candle room	1	W	31	10	3.0	9	W	59	16	3.7
	2	W	62	16	2.5	10	W	44	14	3.1
	3	W	92	24	3.8	11	W	61	17	3.6
	4	W	71	16	4.4	12	W	57	16	3.5
	5	W	35	17	2.0	13	W	74	17	4.3
	6	W	48	17	2.8	14	W	13	15	0.86
	7	W	84	17	4.9	15	B	16	15	1.06
	8	R	20	16	1.25					
	Average		*55.3*	*16.6*	*3.08*	*Average*		*46.2*	*15.7*	*2.86*
100 ft. candle room	16	W	77	24	4.2	20	W	72	14	4.0
	17	W	51	21	2.4	21	W	64	17	3.7
	18	W	64	17	3.7	22	W	48	15	3.20
	19	R	49	15	3.26	23	W	10	7	1.42
						24	B	23	15	1.53
	Average		*60.2*	*19.2*	*3.39*	*Average*		*43.4*	*13.6*	*2.77*
1000 ft. candle room	25	W	45	17	2.6	29	W	23	14	1.6
	26	W	36	14	2.5	30	W	94	24	3.8
	27	W	89	17	5.2	31	W	15	12	1.2
	28	R	43	15	2.86	32	W	96	29	3.3
						33	W	43	17	2.5
						34	W	40	16	2.5
						35	W	52	14	3.7
						36	W	15	15	1.0
						37	B	32	15	2.13
	Average		*53.2*	*15.7*	*3.15*	*Average*		*45.5*	*17.3*	*2.41*

Chart V.—Record of the growth of guinea pigs.

b. Guinea Pigs.—The 36 guinea pigs placed in the experimental rooms apparently developed scurvy so that for the purpose of this experiment the group was unsatisfactory. The healthy guinea pigs placed in the experimental rooms were given dog chow and were not allowed lettuce or similar food because of the desire to avoid an exposure of the food to light. As previously stated, the animals remained healthy for a period of ten to fifteen days, after which time they developed shaggy hair, some muscular weakness, and subsequently a marked fragility of the bones. Litter mates in all three rooms became sick at approximately the same time. Since the pigs received no other food than the dog chow, we were of the opinion that they developed vitamin C deficiency. Analysis of Chart V shows that the guinea pigs were in the experimental rooms approxi-

mately two weeks. The brief exposure period and the vitamin C deficiency present make comparison of these animals unsatisfactory. The average daily weight gain during their growth period was 3.08 grams for the male pigs in the 3 foot-candle room and 3.39 and 3.15 grams respectively for the male pigs in the 100 and the 1000 foot-candle rooms. Similar small differences were noted in the female pigs, but the gain was greatest in the 3 foot-candle room and smallest in the 1000 foot-candle room. A comparison of the litter mates showed wide variations in the average weight gain (Chart V).

	MALE				FEMALE			
	Animal number	Total weight gain (grams)	Growing days	Average daily weight gain (grams)	Animal number	Total weight gain (grams)	Growing days	Average daily weight gain (grams)
3 ft. candle room	*1*	85	29	2.93	*17*	34	33	1.03
	2	62	33	1.87	*18*	154	81	1.90
	3	77	33	2.33	*19*	84	51	1.64
	4	138	43	3.20	*20*	93	51	1.82
	5	169	43	3.93				
	6	137	43	3.18				
	7	111	43	2.71				
	8	121	43	2.81				
	9	48	33	1.42				
	10	145	43	3.39				
	11	66	33	2.00				
	12	159	80	1.98				
	13	192	80	2.40				
	14	175	80	2.18				
	15	118	50	2.36				
	16	159	50	3.18				
	Average		*47.3*	*2.61*	*Average*		*54*	*1.59*
100 ft. candle room	*21*	69	33	2.09	*34*	35	33	1.06
	22	62	33	1.87	*35*	39	33	1.18
	23	65	33	1.96	*36*	34	32	1.03
	24	85	43	1.97	*37*	130	81	1.72
	25	76	43	1.76	*38*	154	81	1.90
	26	62	43	1.44	*39*	116	82	1.41
	27	79	33	2.39	*40*	86	52	1.65
	28	139	43	3.73	*41*	80	52	1.53
	29	47	33	1.31				
	30	309	80	3.86				
	31	170	81	2.09				
	32	161	51	3.15				
	33	137	51	2.68				
	Average		*46.1*	*2.33*	*Average*		*55.7*	*1.43*
1000 ft. candle room	*42*	49	33	1.48	*57*	28	33	0.84
	43	56	33	1.69	*58*	29	33	0.87
	44	77	43	1.79	*59*	25	24	1.04
	45	128	43	2.97				
	46	76	43	1.76				
	47	91	43	2.11				
	48	116	43	2.69				
	49	58	33	1.75				
	50	112	43	2.60				
	51	62	33	1.87				
	52	145	83	1.75				
	53	178	83	2.14				
	54	171	83	2.06				
	55	153	53	2.88				
	56	127	53	2.40				
	Average		*49.6*	*2.12*	*Average*		*30*	*0.91*

Chart VI.—Record of the growth of albino rats.

c. *Rats.*—The 56 rats proved to be satisfactory animals in this experiment. These animals remained in good health throughout the experimental period, and most of them were sacrificed after being in the experimental rooms approxi-

mately fifty days. Chart VI gives a comparison of the growth of the 41 male rats. It reveals that the average daily weight gain of the male rats in the 3 foot-candle room (2.61 grams per day) exceeded that of the male rats in the 100 foot-candle room (2.33 grams) and that of the male rats in the 1000 foot-candle room (2.12 grams). A similar trend is noted in the small group of female rats. The average daily weight gain in the 100 and the 1000 foot-candle rooms was less than that in the 3 foot-candle room. A comparison of litter mates likewise reveals a tendency for litter mates in the 3 foot-candle room to gain more per day than litter mates in the 100 and the 1000 foot-candle rooms. The average daily weight gain of each rat is shown graphically in Chart VII. From a study of this chart it is evident that few animals in the 1000 foot-candle room gained as much as the animals in the 100 and the 3 foot-candle rooms. A study of the growth curves in Chart VIII shows the growth of animals was relatively uniform throughout the experimental period. From this chart one would assume that the beneficial or harmful effect of varying quantities of light was not evident in any particular period of the experiment.

d. Chickens.—A few chicks died in the early days of the experiment, probably because of relatively low temperatures for unfeathered birds, and the hens were discarded from the experiment when they were identified. The remaining 56 cockerels were exposed for approximately eighty-three days and then sacrificed. The health of all birds was good throughout the experiment.

A comparison of the growth of the chickens is tabulated in Chart IX. The cockerels in the 3 foot-candle room gained less (7.89 grams) than those in the 100 foot-candle room (8.07 grams) and more than those in the 1000 foot-candle room (7.47 grams). Chart IX indicates that the different levels of illumination in the experimental rooms did not produce significant variation in the rate of growth of cockerels. Chart X reveals the growth curves of the cockerels to be similar.

B. Specific Observations of Animals

1. Eyes.—The eyes of the animals were frequently observed to study the effect of various quantities of light on the conjunctivae, the wink reflex, and the fundi. Examination of the eyes was done carefully because any possible damage due to light could be more readily detected in the eye than elsewhere.

The conjunctivae of the animals in all rooms were normal to gross examination. There was no tendency of any of the animals to blink frequently or to close their eyes in an attempt to avoid the light.

Fundoscopic examinations were done on a number of rabbits, and several were found to have increased pigmentation resembling chorioretinitis. These animals, however, were not examined until after they had been exposed to the light, and the pigment may have been present prior to their exposure to light in the experimental rooms. In two rabbits a clouding developed in the media, making a clear view of the fundi impossible. Slit-lamp examination of one localized the difficulty in the lens; the other animal died of infectious diarrhea before examination. The fundi of two litters of rabbits, six animals, were studied before their exposure to light in the experimental rooms. Although all six rabbits were albinos, a few pigmented hairs were found on the tips of their tails and on their ears, and some of the group were found to have partially

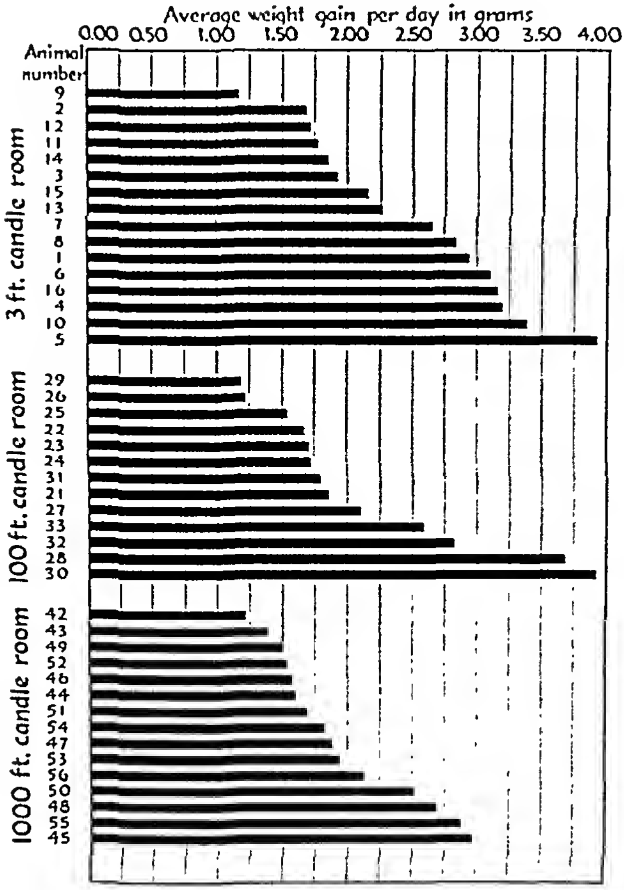


Chart VII.—Average daily gain in weight of rats.

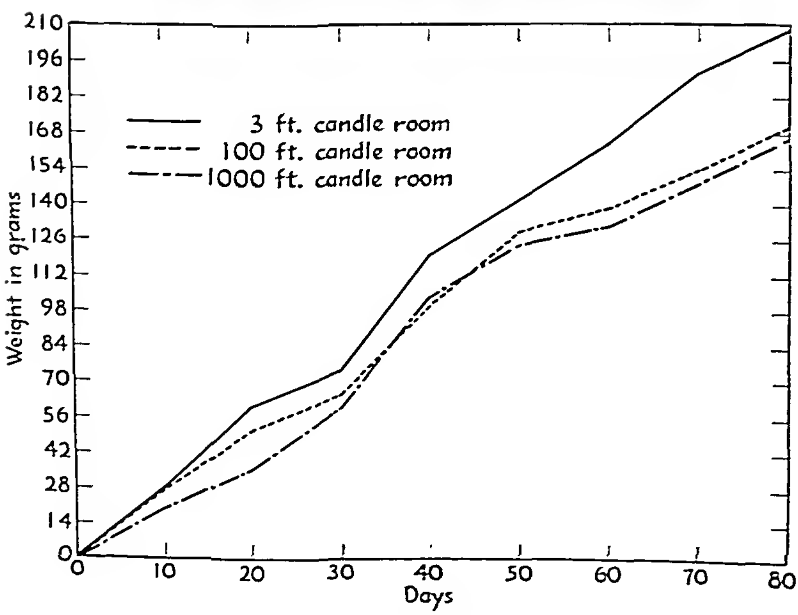


Chart VIII.—The growth curve of rats.

pigmented fundi. The amount of pigment in the fundi did not increase in any of these rabbits after their exposure to light in the 3, the 100, or the 1000 foot-candle room. It was noted that normal black rabbits have a large amount of pigment in their fundi; hence the presence of pigment after exposure to light need not be considered abnormal.

The examining ophthalmologist, Dr. Frances MacCraken, could find no evidence that the eyes of the rabbits were injured by high levels of illumination, but further study on this subject should be done. The eyes were removed for histologic examination.

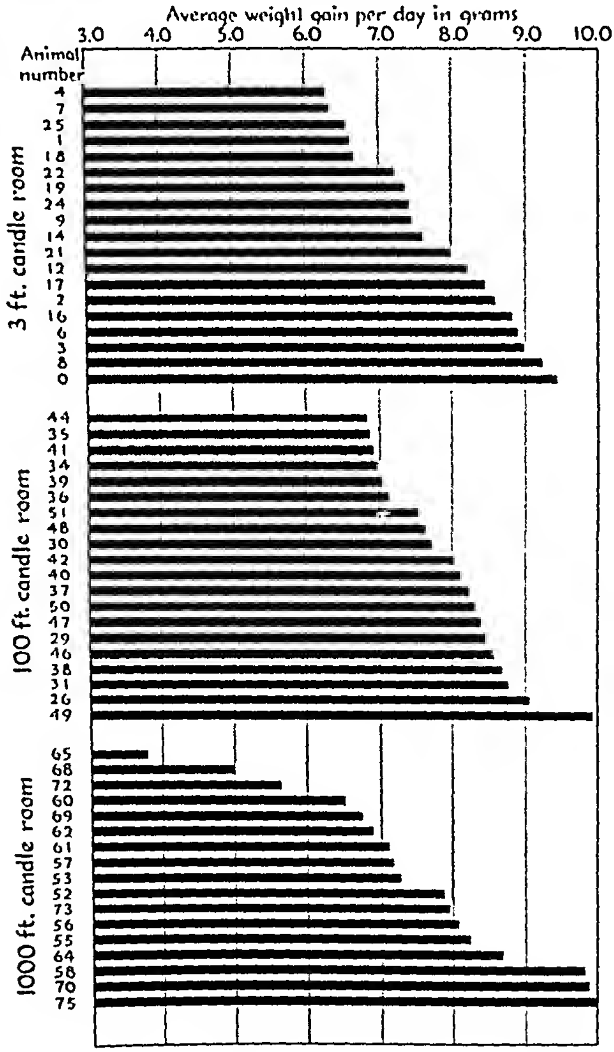


Chart IX.—The average daily gain in weight of cockerels.

2. *Hair and Feathers.*—The hair and the feathers of the experimental animals in the various rooms were compared. No differences could be detected in the texture, the length, the thickness, and the color of the hair or the feathers. The hair of the shorn rabbits and guinea pigs, however, was found to grow most rapidly in the 1000 foot-candle room and least rapidly in the 3 foot-candle

room (Figs. 1, 2). Many rabbits were repeatedly shorn to check this observation. The variation was constant in litter mates. Numerous biopsies of the shorn areas were taken for histologic study.

3. *Laboratory Procedures.*—Only a few laboratory studies were made. Hemoglobin determinations and blood smears were made from a few rats, a few rabbits, and some chickens. Both hemoglobin levels and blood smears were found to be normal and comparable in animals from all the experimental rooms.

C. *Autopsy Studies.*—Autopsies were done on all animals. The majority were sacrificed at the end of the experimental period, but a few died in the course of the experiment.

1. *Gross Studies.*—Rabbits and rats showed no significant gross variations; occasionally a few small areas of bronchopneumonia were noted. Examination of guinea pigs revealed extensive hemorrhages into the muscles of the hind legs and extreme fragility of the bones. The remainder of the organs were essentially normal to gross inspection. The chickens were found to be normal on gross examination.

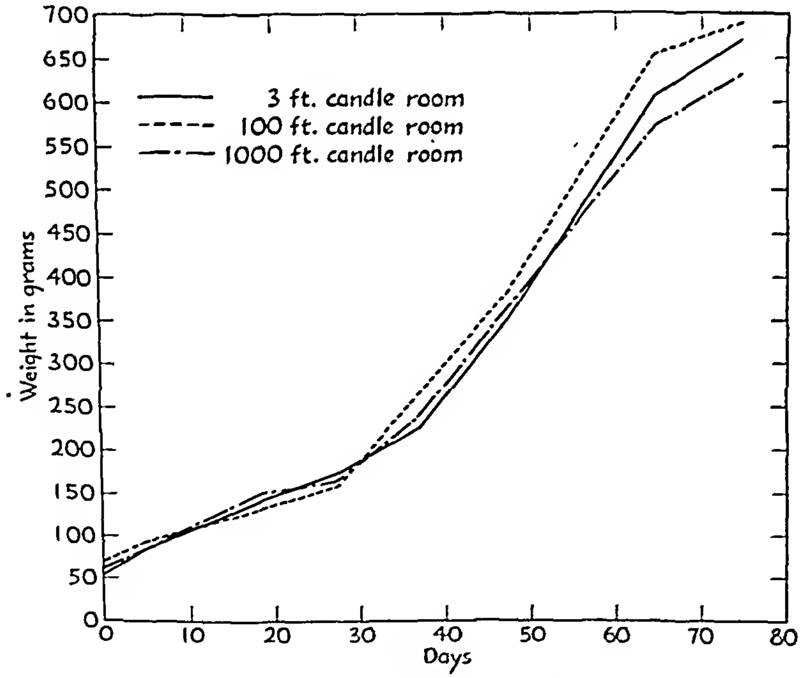


Chart X.—The growth curve of cockerels.

2. *Microscopic Studies.*—Sections from all organs were placed in 10 per cent formalin, sectioned, and stained with hematoxylin and eosin. Pieces of bone (the femurs were usually selected) were decalcified for microscopic study, and skin sections and skin biopsies were prepared from all animals. Examination of the prepared slides did not reveal any significant changes in the organs that one would not find in the study of control animals. Examination of the bones and the muscles of guinea pigs revealed findings similar to those previously described in experimental scurvy.[32] Skin biopsies taken from rabbits

in the experimental rooms revealed a few lymphocytes around some of the vessels of the corium. The number and the distribution of the lymphocytes did not vary according to the exposure of the animals to different intensities of light.

V. DISCUSSION

A. Health.—Physicians frequently advise increased exposure to artificial light and sunlight, but the evidence supporting the beneficial effects of light has been questioned (Clausen).[6] Although many clinical and some laboratory experiments call attention to the value of light, by which are most often meant rays of less than 3300 angstroms, most clinical and laboratory studies have shown that man and animals are able to continue in good health under restricted amounts of light if they are provided adequate diets.

Pearce and Van Allen[30] found evidence that the health of rabbits was not impaired by darkness, but that the rabbits exposed to constant light gained more weight than the unexposed group. From post-mortem observations they found no evidence that spontaneous lesions occurring in the experimental animals were related to the amount of light to which they had been exposed. Mackay[27] could find little evidence that infants exposed to mercury vapor quartz lamps grew faster or were in better health than a control group of children.

The anemic appearance of those living and working in poorly lighted homes and factories does not always indicate poor health. Little anemia has been found in animals working in mines.[16]. Rats placed in dark rooms were found to have a decreased number of blood platelets, but their general health remained good.[8] Laboratory studies on animals subjected to long periods of darkness have shown very little variation in the hemoglobin percentage or in the red cell count if the diet was adequate. Dogs placed in darkness for brief periods have shown an initial decrease in red blood corpuscle counts but a subsequent quick return to normal. Clinicians, on the other hand, have observed a rapid increase in hemoglobin and in red blood cells in anemic individuals exposed to solar and quartz mercury lamp radiations. Miles and Laurens[28] have shown that irradiations of anemic animals with ultraviolet rays are followed by an increase in the cellular elements and in hemoglobin; in this study, however, the changes were probably minimal and insignificant.

The general belief that increased quantities of light perhaps produce better health has led to the use of artificial light in the treatment of wounds and in the prevention of infections. Sunlight has been found to retard the growth of bacteria,[11] and ultraviolet radiation is advocated to reduce the frequency of infections in hospital operating rooms.[19, 23] Hill and Clark,[22] however, were unable to show that ultraviolet rays are beneficial to rats infected with pneumococci. In recent studies Buchbinder, Solowey, and Phelps[5] report the effect of various types and quantities of light on the growth of several strains of streptococci. They found bright sunlight to be a more powerful lethal agent on growing bacteria than daylight or artificial light. Their experiment indicates that the lethal action of light on bacteria is somewhat proportionate to the quantity of light and is related to the spectral quality of the light. The authors suggest that the suppression of the growth of bacteria by light may play an important role in the control of upper respiratory tract infections.

Fig. 1.—Litter mates from the 3, the 100, and the 1000 foot-candle rooms. Photograph taken five days following shaving.

Observers disagree concerning the effect of light on the healing of experimental wounds. Coburn and Cowles[7] found that experimental wounds of rats heal as well in darkness as in sunlight. On the other hand, Leo and Vaucher[26] and other experimenters as well have shown that wounds exposed to sun heal better than those covered. Crile[9] found artificial light from Mazda lamps a helpful adjunct in the treatment of wounds, but the beneficial effects in this instance could be due to the increased heat. Clausen,[6] after carefully considering controlled clinical studies and laboratory experiments, expressed doubt that light favors growth and promotes resistance to infections.

B. Growth.—Most investigators have found evidence that sunlight and artificial light stimulate the rate of growth of man and of experimental animals. This observation has proved of practical value in that chicks are often exposed to artificial light during their growth period.[29] In reviewing the reported experiments on the comparative rate of growth, one finds that investigators have usually compared the growth of animals in darkness or in minimal light devoid of ultraviolet rays to the growth of animals exposed to sunlight or to artificially produced ultraviolet rays.

Wade Brown[4] placed groups of normal rabbits in an experimental room in which neon tubes were added to produce 20 to 50 foot-candles of light. The rabbits in this room gained more weight than other rabbits grown in a dark room and more than rabbits grown in sunlight. In this experiment two groups of animals were exposed to ultraviolet rays while the quantity of light in the group exposed to sunlight is unknown. In comparable studies albino rats grown in darkness were found to grow more slowly than rats grown in sunlight,[25] and rabbits exposed to 425 foot-candle illumination were found to gain more than rabbits grown in dark rooms and in control rooms.[31] Bovie[3] noted that chickens that were raised in sunlight passed through a glass roof (probably devoid of ultraviolet rays) grew poorly compared to chickens raised in uninterrupted sunlight, and Borissow[2] found that dogs and rabbits grew better in adequate than in dim light.

Degkwitz[14] found that puppies raised in darkness appeared to be as healthy as control animals, but they showed a measured impoverishment in total ash, calcium, phosphorus, and magnesium. In a study of the growth of rats on deficient diets, Goldblatt and Soames[17] found that the rats irradiated with quartz mercury lamps grew more than the nonirradiated animals.

Northrop[28] calls attention to the fact that most reported experiments fail to show the effect of various quantities of light on the rate of growth of experimental animals. He exposed imagoes to varying intensities of light and discovered that there was no change in the rate of growth in intensities up to 600 meter candles, but he found that above that level the length of life of the imagoes decreased rapidly. After exposing flies to 10,000 meter candles, Northrop found their life span to be only 3.9 days compared to an average of 13.5 days for flies kept in the dark. He was unable to find evidence, however, that the absence of light in 230 generations had any effect on the duration of life or on the ability of the flies to resist infections. Higgins and Sheard[21] noted that frogs exposed to quartz mercury lamps were stimulated in the first twenty-four hours but that subsequently they showed a retardation of growth.

In a study of 100 healthy children Frank[16] was unable to correlate the growth periods with temperature, precipitation, or the amount of sunlight to which the children were exposed. Clinical studies are unsatisfactory because of the many factors beyond the control of the observer.

In our study we could find no evidence that an increased amount of light devoid of ultraviolet rays promotes growth. High levels of illumination, however, apparently produce no unfavorable influence on the health of experimental animals.

Fig. 2.—Litter mates from the 100 and the 1000 foot-candle rooms four days after shaving.

C. Eyes.—Artificial light rarely produces clinical symptoms or pathologic effects, but light improperly directed may be uncomfortable to the eye and in a few instances produce severe damage. Overstimulation produced by a constant bright light is thought to result generally in only physiologic fatigue and only very seldom in any detectable tissue damage.[12] The action of light upon the eye has been shown to be dependent on its wave length and on its intensity.[12] The shorter radiations, 3150 angstroms or less, are largely absorbed by the cornea or the lens while the radiations of the visible spectrum are more likely to be transmitted to the retina. Conjunctival and corneal lesions have been noted when the eye was exposed to strong radiations from artificial illuminants,

as mercury quartz lamps, or when the eye was exposed to reflected sunlight. Very long wave lengths may produce damage to all layers of the eye, the damage varying in degree with the intensity of the incident illumination and with the boundaries of transmission and absorption found to be different in different individuals. It is believed that the human eye is more transparent in the visible range than that of most nocturnal animals.

D. Sexual Maturity.—It is a common belief that natives in the tropics mature earlier than individuals living in temperate zones. In a careful clinical study Sundstroem[32] found that Anglo-Saxon girls in the tropics reached maturity at approximately the same age as girls in England; hence he believes that early maturity is a racial characteristic. Experimental studies, however, indicate that exposure to increased quantities of light produces early maturity and an increased size of the endocrine glands.

Fiske[15] raised groups of immature female rats in darkness, ordinary light, and continuous light. She noted that the immature female rats kept in light from birth or from the twenty-first day of life came into sexual maturity somewhat earlier than rats grown in ordinary laboratory light and much earlier than rats grown in complete darkness. In her group of males Fiske found at autopsy that the pituitaries, the testes, and the seminal vesicles were largest in the rats exposed to the maximum quantity of light. The difference in size was seen to be be greatest at 150 days; after 150 days the weights of the organs became comparable to the weights of organs from rats raised in dark rooms. Fiske points out that her study indicates a stimulating effect by light on the pituitaries of both male and female rats. Lack of light, in her opinion, favors a decrease in follicle-stimulating hormone and an increase in luteal hormone in the female. Similar conclusions were reached by Dawson[10] in a study of cats and by Benoit;[1] in the latter's study the testicles of immature ducks showed a rapid increase in size proportionate to the amount of illumination to which they were exposed.

In our study no careful observations were made to determine the relative time of development of maturity of animals exposed to different quantities of light. At post-mortem careful studies of the organs of the endocrine system were not made, but no gross deviations were seen in the various rooms.

E. Hair Growth.—An increased rate and amount of hair growth has been noted on the arms of attendants working in solariums. Brown[4] found that shorn albino rabbits exposed to neon lights showed an active proliferation of hair follicles, while rabbits living in the dark showed delayed, incomplete, or irregular growth. He attributed the effect to ultraviolet radiations.

Sundstroem,[32] after carefully measuring and weighing his clipped hair and nails, found that they grow 10 per cent more slowly in the tropics. He indicated that a slower rate of growth would be expected in all furry animals in warm climates.

We were able to confirm the findings of Brown[4] in that our shaved animals showed a more rapid hair growth when exposed to increased quantities of light. We did not, however, find a proliferation of hair follicles in the sections of skin taken by biopsy or obtained at autopsy. We believe the stimulus for increased hair growth may be central rather than peripheral.

F. Post mortems.—Careful post-mortem studies were reported by Pearce and Van Allen[31] of animals exposed to mercury quartz vapor lamps. The

organs of rabbits in the light rooms weighed less than those of rabbits in the control rooms. Small lesions noted in the organs were considered insignificant. Other studies of animals after exposure to light showed no differences in the organs of animals exposed to various quantities of light.

At the autopsies of the animals in our experiment gross inspection showed no differences in the organs of animals from the 3, the 100, and the 1000 foot-candle rooms. In a few animals small lesions were found in some organs, but they were irregular in distribution and did not appear to have any relation to the amount of light to which the animals had been exposed. Examination of slides prepared from the different organs showed them to be no different from the organs of control animals. One could conclude that varying quantities of light produce no obvious tissue damage.

VI. SUMMARY

Groups of rats, rabbits, guinea pigs, and chickens were exposed to different intensities of light in three experimental rooms. The animals received unlimited quantities of similar food in each room, and all were exposed to light an equal length of time daily. All animals were observed frequently to note any difference in behavior due to exposure to various amounts of light, and each animal was weighed frequently.

Animals were sacrificed at the end of an arbitrarily chosen experimental period if they had not died of an intercurrent illness. Autopsies were performed upon all animals, and tissues were obtained for microscopic study.

The health of the animals in the 3, the 100, and the 1000 foot-candle rooms was approximately the same. No differences in behavior of the animals could be detected, and the gain in weight of the various animals in the different experimental rooms was approximately the same. There was a noticeably increased rate of hair growth in animals exposed to higher intensities of light. Autopsy examinations revealed no unusual differences in the organs of animals exposed to various quantities of light.

REFERENCES

1. Benoit, Jacques: Influence de la lumière naturelle sur la croissance testiculaire chez le canard au cours de la reprise sexuelle saisonniere (avec demonstrations), Compt. rend. Soc. de biol. 120: 131, 1935.
2. Borissow, P.: Ueber den Einfluss des Lichtes und der Dunkelheit auf die Zusammensetzung des Blutes, Ztschr. f. diatat. u. physik. Therap. 5: 337, 1900.
3. Bovie, W. T.: Physiological Effects of Radiation, Electromagnic Spectrum, Boston M. & S. J. 192: 1035, 1925.
4. Brown, Wade H.: Influence of Light Environment on Growth and Nutrition of Normal Rabbits With Special Reference to Action of Neon Light, J. Exper. Med. 48: 31, 1928.
5. Buchbinder, Leon; Solowey, Mathilde; and Phelps, Earle B.: Studies on Microorganisms in Simulated Room Environments; Survival Rates of Streptococci in Presence of Natural Daylight and Sunlight, and Artificial Illumination. J. Bact. 42: 353, 1941.
6. Clausen, Ethel M. Luce: Therapeutic Value of Radiation, Medicine 8: 419, 1929.
7. Coburn, John Wesley, and Cowles, Dorence Sheldon: A Study of the Effect of Radiation on Wound Healing, Yale Univ. Med. Thesis, 1925.
8. Cramer, W., and Drew, A. H.: The Effect of Light on the Organism, Brit J. Exper. Path. 4: 271, 1923.
9. Crile, George W.: The Use of Electric Light and Hypochlorous Acid in the Treatment of Wounds, Surg., Gynec. & Obst. 23: 496, 1916.
10. Dawson, Alden B.: Early Estrus in Cat Following Increased Illumination, Endocrinology 28: 907, 1941.
11. Downes, A., and Blunt, T. P.: Researches on the Effect of Light Upon Bacteria, Proc. Roy. Soc., London 26: 488, 1877.

12. Duke-Elder, W. S. D.: Pathological Action of Light Upon Eye: Action on Outer Eye; Photophthalmia, Lancet 1: 1137, 1188, 1250, 1926.
13. Duke-Elder, W. S. D.: Recent Advances in Ophthalmology, Lancet 2: 922, 1927.
14. Degkwitz, R., et al.: Ueber Einflüsse der Ernährung und der Umwelt auf wachsende Tiere. Ein Beitrag zur allgemeinen Prophylaxe und Hygiene des Säuglingsund Kleinkindesalter. I., Ztschr. f. Kinderh. 37: 27, 1924.
15. Fiske, Virginia M.: Effects of Light and Darkness on Activity of Pituitary of Rat, Proc. Soc. Exper. Biol. & Med. 40: 189, 1939.
16. Frank, H.: Seasonal Growth in Infants, Arch. f. Kinderh. 75: 1, 1923.
17. Goldblatt, H., and Soames, K. M.: The Effect of Radiation With the Mercury Vapour Quartz Lamp on the Growth of Rats Fed on a Diet Deficient in the Fat-Soluble Growth-Promoting Factor, Lancet 2: 1321, 1922.
18. Grober, and Sempell, O.: Die Blutzusammensetzung bei jahrelanger Entziehung des Sonnenlichtes, Deutsches Arch. f. klin. Med. 129: 305, 1919.
19. Hart, Deryl: Sterilization of Air in Operating Room With Bactericidal Radiation; Results From Nov. 1, 1938 to Nov. 1, 1939, With Further Report as to Safety of Patients and Personnel, Arch. Surg. 41: 334, 1940.
20. Hart, E. B., Steenbock, H., Lepkovsky, S., and Halpin, J. G.: The Nutritional Requirements of Baby Chicks. III. The Relation of Light to the Growth of the Chicken, J. Biol. Chem. 58: 33, 1923.
21. Higgins, G. M., and Sheard, C.: Effects of Ultraviolet Radiation on the Early Larval Development of Rana Pipiens, J. Exper. Zool. 46: 333, 1926.
22. Hill, C. M., and Clark, J. H.: Effect of Ultraviolet Radiation on Resistance to Infection, Am. J. Hyg. 7: 448, 1927.
23. Kraissl, C. J., Cimiotti, J. G., and Meleney, F. L.: Considerations of Use in Ultraviolet Radiation in Operating Rooms, Ann. Surg. 111: 161, 1940.
24. Laurens, Henry: Physiological Effects of Irradiation, Physiol. Rev. 8: 1, 1928.
25. Laurens, Henry, and Sooy, J. W.: The Effect of Light on the Growth of the Albino Rat, Proc. Soc. Exper. Biol. & Med. 22: 112, 1911.
26. Leo, G., and Vaucher, E.: Les modifications histologiques des plaies de guerre insolees, Compt. rend. Soc. de biol. 81: 138, 1918.
27. Mackay, Helen M. M., and Newbold, E. M.: Artificial Light Therapy in Infancy; Comparison of 66 Cases Treated With Mercury Vapour Quartz Lamp and 137 Control Cases; Statistical Examination of Weight Curves, Arch. Dis. Childhood 2: 231-248, 1927.
28. Miles, A. L., and Laurens, Henry: Physiological Action of Darkness, Daylight and of Carbon Arc Radiation; Effects of Darkness on Physical Characters of Blood of Dogs, Am. J. Physiol. 75: 443, 1926.
29. Northrop, J. H.: Influence of Intensity of Light on Rate of Growth and Duration of Life of Drosophila, J. Gen. Physiol. 9: 81, 1925.
30. Pearce, Louise N., and Van Allen, Chester M.: Effects of Light on Normal Rabbits, With Special Reference to Organic Reaction; Clinical and Post-Mortem Observations, J. Exper. Med. 44: 447, 1926.
31. Pearce, Louise N., and Van Allen, Chester M.: Effects of Light on Normal Rabbits, With Special Reference to Organic Reaction; Organ Weights, J. Exper. Med. 44: 461, 1926.
32. Sundstroem, E. S.: Studies on the Adaptation of Albino Mice to an Artificially Produced Tropical Climate. I. Effect of the Various Factors Composing a Tropical Climate on Growth and Fertility of Mice, Am. J. Physiol. 60: 397, 1922.
33. Wolbach, S. Burt, and Howe, Percy R.: Intercellular Substances in Experimental Scorbutus, Arch. Path. & Lab. Med. 1: 1, 1926.

UREASE ACTIVITY OF PROTEUS AND SALMONELLA ORGANISMS*

W. W. Ferguson, Ph.D., and A. E. Hook, Ph.D., Lansing, Mich.

One of the most important details of routine enteric bacteriology is the separation of nonpathogens from pathogens in the process of cultural elimination. It is usual for bacteriologists to transfer isolated colonies from differential plate media to triple or double sugar slants and judge from the reaction of the slants whether or not the organisms should be studied further. In the elimination process the greatest trouble to the worker results from those organisms which produce an alkaline surface on the slants, an acid butt and gas.

There are several possibilities as to the identity of such an organism. It may belong to the paracolon group, consisting of slow lactose- and sucrose-fermenting organisms with general coliform characteristics. It may belong to the proteus group whose members are nonlactose-fermenting, display a variable ability to ferment sucrose, and whose swarming tendencies are inhibited on differential media. It may belong to the genus Salmonella.

Among those working with animal fecal pathogens, the problem of separation of Proteus bacteria from Salmonella-like organisms is particularly burdensome because of the many Proteus strains encountered.

A test for swarming will prove of considerable value in discovering Proteus strains but, unfortunately, such a test is not entirely reliable. A small percentage of Proteus organisms have no ability to swarm; some Proteus strains are not highly motile and give an inconclusive reaction on two per cent agar. It has been our experience that some Salmonella strains on moist agar simulate the swarming activity of Proteus.

It is known that the urease activity of Proteus organisms and the nonurease activity of Salmonella provide a method of separating the commonly encountered members of the genus Proteus from organisms of the Salmonella group.

Numerous investigators have attested to the urea-decomposing ability of Proteus organisms. Of particular interest are the observations of Moltke[1] and of Rustigian and Stuart[2] who have made extensive studies on urea decomposition. Moltke examined 194 strains of maltose-fermenting and nonmaltose-fermenting strains of Proteus and found that all attacked urea. In 1941 Rustigian and Stuart[2] reported that all members of the genus except *Proteus hydrophilus*, *Proteus ichthyosmius*, and *Proteus bombycis* were capable of decomposing urea with formation of ammonia and subsequent production of alkali. They agreed with St. John-Brooks and Rhodes[3] that *Proteus hydrophilus* and *Proteus ichthyosmius* should be excluded from the genus. Rustigian and Stuart would also exclude *Proteus bombycis* from the genus for its inability to decompose urea.

The paracolon group of organisms has apparently not been studied for urease activity except by Ashworth[4] who tested 30 paracolon strains and found them unable to attack urea.

---

*From the Bureau of Laboratories, Michigan Dept. of Health.

Received for publication, May 5, 1943.

According to Bergey,[3] organisms of the Tribe Salmonelleae do not hydrolyze urea.

In a study on Salmonelliasis in infants, Hormaeche and Peluffo[5] described a simple medium consisting of Simmon's agar plus urea which they employed for differentiating Proteus and Salmonella organisms. They stated that the latter exert no action on the urea.

In our search of the literature we have been unable to find a comprehensive list of Salmonella strains which have been examined for urease activity. It is the purpose of this paper to supply such a list in the hope that other workers will be encouraged to use a test for decomposition of urea as an aid in enteric bacteriology.

MEDIA

Three media were used in testing the organisms listed in this paper.

Formula 1 was the synthetic medium of Rustigian and Stuart,[2] containing 2 per cent urea (Baker's. C.P.),* 0.01 per cent yeast extract (Difco), and M/15 primary and secondary phosphate buffers (Sorensen) in distilled water to give a final pH of 6.8. Sterilization was obtained by filtration through Seitz pads.

Formula 2 was devised in our laboratory. Its ingredients were as follows: 2 per cent urea (Baker's, C.P.), 0.1 per cent KH_2PO_4, 0.1 per cent K_2HPO_4, 0.5 per cent NaCl and 1.0 per cent ethyl alcohol (95 per cent). The pH was adjusted to 7.0. Sterilization was accomplished by filtration through a Seitz pad.

Both media were dispensed in 3 to 5 c.c. amounts in sterile 5 × ⅝ inch culture tubes.

Formula 3 was the solid medium of Ashworth[4] dispensed in the form of slopes. Its ingredients were: agar 2.5 per cent, distilled water, K_2HPO_4 0.1 per cent, $CaCl_2$ 0.01 per cent, Mg So_4. $7H_2O$ 0.01 per cent, NaCl 0.01 per cent, $FeCl_3$ 0.001 per cent, beef extract (Difco)† 0.5 per cent, brom thymol blue (1.5 per cent) 1 per cent, and urea 2.0 per cent. All ingredients, except urea, were dissolved in distilled water, and the base was sterilized by a temperature of 121° C. for twenty minutes. Sufficient 20 per cent urea solution was added to make a final concentration of 2 per cent urea.

METHOD OF TESTING

Organisms under test for urease activity were inoculated by loop from a 24-hour growth on veal infusion agar slants to the liquid media of Formulas 1 and 2 and to the slanted surface of Formula 3. After incubation for 24 hours at 37° C. the liquid media were tested for formation of $(NH_4)_2CO_3$ by the addition of several drops of brom thymol blue indicator. An intense blue color was accepted as evidence of the breakdown of urea with formation of alkali. The solid slants of Ashworth's medium (Formula 3) were observed for change in reaction 24 hours after inoculation of test organisms.

TEST ORGANISMS

Thirty strains of Proteus from animal and human sources were tested for urease activity. Their reactions in urea media are given in Table I. Seven of

*Rustigian and Stuart specified Merck's urea.
†Ashworth specified Lemco beef extract.

the cultures came originally from the American Type Culture Collection; however, of these, *Proteus hydrophilus* I and II did not possess the characteristics of this species. *P. hydrophilus* I gave the biochemical reactions of *Proteus vulgaris*, with the exception of rapid gelatin liquification; *P. hydrophilus* II resembled *Proteus mirabilis* in its reaction. Both organisms produced swarming on 2 per cent agar. The Proteus strains from animal sources were obtained from the collection of Mr. C. W. Darby of Michigan State College.

TABLE I

REACTION OF PROTEUS ORGANISMS IN THREE UREA MEDIA

CULTURES	SOURCE	FORMULA 1 (RUSTIGIAN AND STUART)	FORMULA 2	FORMULA 3 (ASHWORTH)
S 779 P. mirabilis	Dog	Alkaline	Alkaline	Alkaline throughout
S1002 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
P. hydrophilus I*		Alkaline	Alkaline	Alkaline throughout
P. hydrophilus II*		Alkaline	Alkaline	Alkaline throughout
P. ichthyosmius*		Neutral	Neutral	Alkaline surface
S1008 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
S 771 P. mirabilis	Dog	Alkaline	Alkaline	Alkaline throughout
S 911 P. mirabilis	Turkey	Alkaline	Alkaline	Alkaline throughout
S1149 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
S1010 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
S 373 P. mirabilis	Dog	Alkaline	Alkaline	Alkaline throughout
S 983 P. mirabilis	Dog	Alkaline	Alkaline	Alkaline throughout
S1021 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
S1161 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
P. mirabilis I*		Alkaline	Alkaline	Alkaline throughout
P. mirabilis II*		Alkaline	Alkaline	Alkaline throughout
P. ammoniae*		Alkaline	Alkaline	Alkaline throughout
S 599 P. mirabilis	Pig	Alkaline	Alkaline	Alkaline throughout
S1003 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
P. americanus*		Alkaline	Alkaline	Alkaline throughout
S 429 P. mirabilis	Dog	Alkaline	Alkaline	Alkaline throughout
S1144 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
S 777 P. mirabilis	Dog	Alkaline	Alkaline	Alkaline throughout
P 100 P. mirabilis	Monkey	Alkaline	Alkaline	Alkaline throughout
S 926 P. mirabilis	Turkey	Alkaline	Alkaline	Alkaline throughout
Proteus OX19†		Alkaline	Alkaline	Alkaline throughout
Proteus OX K†		Alkaline	Alkaline	Alkaline throughout
Proteus vulgaris (1)	Human	Alkaline	Alkaline	Alkaline throughout
Proteus vulgaris (2)	Human	Alkaline	Alkaline	Alkaline throughout
Proteus vulgaris (3)	Human	Alkaline	Alkaline	Alkaline throughout

*American Type Culture Collection.
†Mich. Dept. Health Stock Culture Collection.

In Table II are recorded the reactions in urea media of thirty-eight strains of Salmonella obtained also from Mr. C. W. Darby. All strains came originally from Dr. P. R. Edwards of the University of Kentucky. Since these organisms had undergone several transfers since their initial typing, their Salmonella characteristics were checked by us. None of the organisms fermented lactose or sucrose, produced indol or liquefied gelatin. All were gram-negative and motile.

In Table III are recorded the reactions in urea media of thirty-seven Salmonella strains obtained from the stock culture collection of the Michigan Department of Health. Many of the organisms were identified by Dr. P. R. Edwards; the identity of the others was vouched for by the curator of stock cultures, Dr. Janet Bourn. All of the cultures were tested, however, for fermentation of lactose and sucrose, production of indol and liquefaction of gelatin,

gram staining qualities and motility. All of the organisms were Salmonella-like in their reactions.

In Table I it will be noted that all Proteus strains show evidence of decomposing urea with the exception of *Proteus ichthyosmius*. This organism produced a neutral reaction in Formula 1 and 2 and an alkaline slant surface in Formula 3, Ashworth's medium. Other Proteus organisms produced a deep blue color throughout Ashworth's medium.

TABLE II

REACTION OF THIRTY-EIGHT SALMONELLA STRAINS IN UREA MEDIA

CULTURE NO.	GROUP	SPECIES	FORMULA 1 (RUSTIGIAN AND STUART)	FORMULA 2	FORMULA 3 (ASHWORTH'S)
284	A	*S. paratyphi* (15)	Neutral	Neutral	Alkaline surface
274	B	*S. typhi murium*, Var. Copenhagen (19500)	Neutral	Neutral	Alkaline surface
278	B	*S. bredeney* (1051)	Neutral	Neutral	Alkaline surface
281	B	*S. schottmuelleri* (A.M.S.)	Neutral	Neutral	Alkaline surface
282	B	*S. abortivoequina* (WII_2)	Neutral	Neutral	Alkaline surface
283	B	*S. derby* (1729)	Neutral	Neutral	Alkaline surface
285	B	*S. typhi murium* (M. 191871)	Neutral	Neutral	Alkaline surface
287	B	*S. california* (cal. 55)	Neutral	Neutral	Alkaline surface
375	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
376	B	*S. schottmuelleri*, Var. Odense	Neutral	Neutral	Alkaline surface
377	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
378	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
380	B	*S. heidelberg*	Neutral	Neutral	Alkaline surface
275	C	*S. muenchen* (425)	Neutral	Neutral	Alkaline surface
276	C	*S. cholerae suis* (1348)	Neutral	Neutral	Alkaline surface
279	C	*S. cholerae suis* Var. Kunzendorf (1713)	Neutral	Neutral	Alkaline surface
286	C	*S. newport* (197)	Neutral	Neutral	Alkaline surface
296	C	*S. bareilly* (K.L.)	Neutral	Neutral	Alkaline surface
290	D	*S. gallinarum*	Neutral	Neutral	Alkaline surface
292	D	*S. enteritidis* (205)	Neutral	Neutral	Neutral throughout
352	D	*S. rostock*	Neutral	Neutral	Alkaline surface
353	D	*S. moscow*	Neutral	Neutral	Neutral throughout
354	D	*S. blegdam*	Neutral	Neutral	Alkaline surface
355	D	*S. berta*	Neutral	Neutral	Alkaline surface
289	E	*S. senftenberg* (5765-1)	Neutral	Neutral	Alkaline surface
293	E	*S. anatum* (M.C.M.1)	Neutral	Neutral	Alkaline surface
294	E	*S. new brunswick*	Neutral	Neutral	Alkaline surface
295	E	*S. give* (316)	Neutral	Neutral	Alkaline surface
372	E	*S. meleagridis*	Neutral	Neutral	Alkaline surface
373	E	*S. give*	Neutral	Neutral	Alkaline surface
288	Additional	*S. kentucky* (18)	Neutral	Neutral	Alkaline surface
291	Additional	*S. worthington* (Minn. 39)	Neutral	Neutral	Alkaline surface
358	Additional	*S. carrau*	Neutral	Neutral	Alkaline surface
298	Additional	*S. minnesota* (Minn. 2)	Neutral	Neutral	Alkaline surface
359	Additional	*S. onderstepoort*	Neutral	Neutral	Alkaline surface
356	Additional	*S. aberdeen*	Neutral	Neutral	Alkaline surface
357	Additional	*S. poona*	Neutral	Neutral	Alkaline surface

It was conjectured that the reaction of *P. ichthyosmius* in Ashworth's medium was due to the breakdown of nitrogenous substances in the meat extract component. All of the Proteus strains were therefore inoculated onto Ashworth's medium minus urea and were found with no exceptions to produce

alkaline slant surfaces but no reaction in the butt. Uninoculated controls were unchanged by incubator temperature.

The possibility of misinterpretation of reactions on this medium was undoubtedly realized by Ashworth[4] who cautioned that only the full range of brom thymol blue (on the alkaline side) denotes a positive result.

TABLE III

REACTION OF THIRTY-SEVEN SALMONELLA STRAINS IN UREA MEDIA

CULTURE NO.	GROUP	SPECIES	FORMULA 1 (RUSTIGIAN AND STUART)	FORMULA 2	FORMULA 3 (ASHWORTH'S)
501	A	*S. paratyphi*	Neutral	Neutral	Neutral throughout
502	A	*S. paratyphi*	Neutral	Neutral	Neutral throughout
504	A	*S. paratyphi*	Neutral	Neutral	Neutral throughout
503	A	*S. paratyphi*	Neutral	Neutral	Neutral throughout
564	B	*S. derby*	Neutral	Neutral	Alkaline surface
554	B	*S. san diego*	Neutral	Neutral	Alkaline surface
571	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
569	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
557	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
556	B	*S. san diego*	Neutral	Neutral	Alkaline surface
555	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
560	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
563	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
562	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
561	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
544	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
558	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
559	B	*S. schottmuelleri*	Neutral	Neutral	Neutral throughout
543	B	*S. typhi murium*	Neutral	Neutral	Alkaline surface
509	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
507	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
506	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
508	B	*S. schottmuelleri*	Neutral	Neutral	Alkaline surface
568	C	*S. newport*	Neutral	Neutral	Alkaline surface
565	C	*S. oranienburg*	Neutral	Neutral	Alkaline surface
570	C	*S. newport*	Neutral	Neutral	Alkaline surface
572	C	*S. oranienburg*	Neutral	Neutral	Alkaline surface
549	C	*S. newport*	Neutral	Neutral	Alkaline surface
546	C	*S. choleraesuis*	Neutral	Neutral	Alkaline surface
545	C	*S. choleraesuis*	Neutral	Neutral	Neutral throughout
550	C	*S. choleraesuis*	Neutral	Neutral	Alkaline surface
553	C	*S. bareilly*	Neutral	Neutral	Alkaline surface
552	C	*S. newport*	Neutral	Neutral	Alkaline surface
567	D	*S. panama*	Neutral	Neutral	Alkaline surface
547	D	*S. enteritidis*	Neutral	Neutral	Alkaline surface
542	D	*S. enteritidis*	Neutral	Neutral	Alkaline surface
551	E	*S. new brunswick*	Neutral	Neutral	Alkaline surface

In the media of Formulae 1 and 2 none of the seventy-five Salmonella cultures listed in Tables II and III gave any evidence of attacking urea. In Ashworth's medium none of the Salmonella cultures gave the deep blue coloration throughout the slant which characterized the growth of Proteus strains on this medium. However, 67 strains produced an alkaline surface. It was considered that these organisms, like *Proteus ichthyosmius*, were incapable of decomposing urea but did produce an alkaline reaction from the breakdown of the meat extract of Ashworth's medium. When the same Salmonella strains which produced an alkaline surface on the urea medium were grown on Ashworth's medium minus urea, they again produced alkali on the surface of the slope.

Rustigian and Stuart[2] tested the reaction of Proteus strains in their medium with and without urea and established that alkalinity resulted only when urea was present. Formula 2, with and without urea, was tested by us with Proteus and Salmonella cultures. An alkaline reaction resulted from the growth of Proteus only when urea was present; no change in the pH of the medium was produced by growth of Salmonella cultures whether or not urea was present.

DISCUSSION

The reactions of thirty Proteus and seventy-five Salmonella strains in urea media were unusually clear-cut. Only *Proteus ichthyosmius* of the thirty Proteus strains failed to decompose urea. None of the Salmonella cultures showed evidence of decomposing urea.

It is true that all of the organisms tested by us were stock cultures or had lived for several generations on artificial media. In addition to these, approximately fifty newly isolated Salmonella strains have been tested for urea decomposition in the laboratories of the Michigan Department of Health during the last eight months. None of the Salmonella cultures attacked urea.

It will not be surprising if one or more Salmonella of the 107 species listed by Kauffmann[7] are found capable of attacking urea. Some strains of *S. Eastbourne*, it will be remembered, vary from the Salmonella characteristics by producing indol; three species of Salmonella liquefy gelatin and a variant of *S. anatum* has been described which ferments lactose. However, the results in this paper indicate that urea-decomposing strains of Salmonella will rarely, if ever, be encountered.

Of the three urea media used our inclination is in favor of the liquid medium of Rustigian and Stuart, or Formula 2. Ashworth's solid medium, while convenient to use, may very well give misleading results to one who is unfamiliar with its reactions with and without urea.

SUMMARY

Of thirty strains of Proteus, *Proteus ichthyosmius* only failed to decompose urea.

None of seventy-five strains of Salmonella showed evidence of attacking urea.

A test for urease activity is recommended as a routine procedure for separation of Proteus and Salmonella organisms.

REFERENCES

1. Moltke, O.: Contributions to the Characterization and Systematic Classification of Bacillus Proteus Vulgaris (Hauser), Copenhagen, 1927, Levin and Munksgaard.
2. Rustigian, R., and Stuart, C. A.: Decomposition of Urea by Proteus, Proc. Soc. Exper. Biol. & Med. 47: 108, 1941.
3. St. John-Brooks, R., and Rhodes, M.: Taxonomy of the Proteus Group, Third International Congress for Microbiology, Report of Proceedings, 167, 1939.
4. Ashworth, P. G.: The Production of Ammonia by Bacteria, M. J. Australia 1: 125, 1942.
5. Bergey's Manual of Determinative Bacteriology, ed. 5, Baltimore, 1939, Williams and Wilkins Co.
6. Hormaeche, E., and Poluffo, C.: Salmonelliasis in Infancy and Its Diagnosis, Puerto Rico J. Pub. Health & Trop. Med. 17: 99, 1941.
7. Kauffmann, F.: Die Baktoriölogie der Salmonella-Gruppe, Copenhagen, 1941, Lovin and Munksgaard.

THE CEPHALIN FLOCCULATION TEST IN JAUNDICE*

PAUL A. KIRSCHNER, M.D., AND STANLEY I. GLICKMAN, M.D., NEW YORK, N. Y.

IN 1938 and 1939 Hanger[1, 2] reported a new laboratory test employed as a diagnostic measure in jaundice of various types. It was an empirical reaction, of incompletely understood mechanism, which depended on the flocculation of a cephalin-cholesterol emulsion in the presence of the serum of patients with active parenchymal disease of the liver. In a series of over 900 normal individuals there was only one weakly positive reaction, while in catarrhal jaundice and acute hepatitis it was found to be positive at the height of the disease. The reaction in obstructive jaundice was negative or only very weakly positive, the latter occurring in cases of prolonged obstruction and/or acute associated hepatitis. The test was found to parallel the activity of the pathologic process in cirrhosis. Hemolytic jaundice gave negative results. However, Hanger[1] listed a number of miscellaneous conditions with or without jaundice, including such diseases as subacute bacterial endocarditis, various types of pneumonia, nephritis, and other infections in which the test was positive in a goodly proportion of instances. Nevertheless, if the clinical picture, corroborative laboratory data, and pathologic findings were carefully studied, and particularly if the test was confined to jaundiced individuals, there was a good indication that the cephalin flocculation reaction was a reliable index of the degree and type of active parenchymal disease of the liver.

In 1940 Hanger and Gutman[3] published the clinical, chemical, and pathologic findings in cases of icterus following arsphenamine therapy. These observers had previously noted that in a minority of patients with postarsphenamine jaundice the cephalin flocculation test gave negative results and that the serum phosphatase activity was elevated, indicating an obstructive intrahepatic type of icterus rather than the usual parenchymatous type. This was confirmed by pathologic examination. It was on the basis of these studies that the cephalin flocculation test was found to have selective value in differentiating obstructive from nonobstructive jaundice.

Since then, several observers have reported their results with the flocculation test, and although their findings occasionally vary from those of Hanger, they all accept the test as a dependable aid in the differential diagnosis of jaundiced conditions. Pohle and Stewart[4] found in a series of 141 patients with hepatic or biliary tract disease that the reaction was positive in varying degrees in most instances. However, they regarded a one- or two-plus reaction as positive. They were impressed with the sensitivity of the reaction in detecting degrees of liver damage rather than its selectivity in distinguishing

*From the Medical and Surgical Services of the Mount Sinai Hospital.
Received for publication, September 6, 1943.

obstructive from nonobstructive jaundice. In their hands false positive reactions were rare. Rosenberg[5] also emphasized the delicacy of the test, for he considered any positive reaction (even as slight as plus-minus) as indicative of liver disease. In employing the procedure as a means of separating obstructive from parenchymatous jaundice, he based his conclusion on the degree of flocculation, rather than on its presence or absence. Nadler and Butler[6] agreed in general with the observations of Hanger. Of particular interest is their finding that four cases of jaundice following arsphenamine therapy all gave negative reactions, thereby confirming the observations of Hanger and Gutman. They employed a cephalin which was obtained from the same source as that used in the present study and suggested that the reagent used by Pohle and Stewart was too sensitive.

Mateer, Baltz, Marion, Hollands, and Yagle[7] reported an extensive comparative laboratory evaluation of hepatic and biliary tract disease which included the cephalin cholesterol flocculation reaction. They noted that the test was at once specific and sensitive and recommended it without reservation. However, since their cases are not grouped from the standpoint of "medical" versus "surgical" jaundice, their results are not strictly comparable to those included in this paper. Nevertheless, they pointed out that although Hanger held that "unripened" cephalin (i.e., that which is not allowed to oxidize by lengthy exposure to air) is more sensitive and more liable to give false positive reactions, the reverse held true in their experiences. Specifically, in 40 normal controls there were 5 one-plus reactions after forty-eight hours with the "unripened" material, while the use of the "ripened" substance gave several times as many positive results, some even two-plus reactions. A more recent report[8] relates the relative value of the commercial substance made by the Difco Company of Detroit (that used in this work), namely, that a one-plus reaction per se had no positive significance.

The present study includes 154 cases of jaundice observed on the wards of the Mount Sinai Hospital from October, 1941 to April, 1943. They have been grouped under the headings of obstructive and hepatocellular jaundice, with particular reference to the differentiation of medical and surgical conditions. The sensitivity of the cephalin used (Difco product) coincided with that in Dr. Hanger's laboratory, and the methods employed in the performance of the test were in accord with his directions. The results of the reaction have been designated as 0, 1 plus, 2 plus, 3 plus, and 4 plus, and readings were made after twenty-four and forty-eight hours. Although the 24-hour reading was considered more sensitive, the interpretation of the test was based upon the 48-hour reading, because this was found more clear-cut and reliable. For the sake of reliability and in view of other clinical, chemical, and pathologic findings, the cases giving a 1-plus reaction were interpreted as negative. Dr. Hanger had suggested that a 1-plus reaction had no definite significance in relation to parenchymatous disease, unless this was noted during recovery or in cirrhosis. In a large number of cases the test was done several times in the course of the disease, and some of the repeated determinations indicate the variation correlated with the dynamics of the pathologic process. The proved cases were confirmed by post-mortem examination or surgical exploration.

The results are summarized in Tables I and II.

TABLE I

OBSTRUCTIVE JAUNDICE

		NEGATIVE		POSITIVE		
CALCULOUS OBSTRUCTION (Common Duct Stone)		0	+	++	+++	++++
Number Cases	33	22	8	3	0	0
Proved	25	16	6	3	0	0
CARCINOMATOUS OBSTRUCTION (Common Duct or Pancreas)						
Number Cases	19	15	1	2	1	0
Proved	16	13	0	2	1	0
CICATRICIAL OBSTRUCTION						
Number Cases	11	5	1	0	3	2
Proved	8	3	1	0	2	2
LYMPH NODE OBSTRUCTION						
Number Cases	5	2	3	0	0	0
Proved	2	1	1	0	0	0
ACUTE CHOLECYSTITIS AND JAUNDICE (No Obstructive Factor Demonstrated)						
Number Cases	5	3	1	1	0	0
Proved	5	3	1	1	0	0
CHRONIC CHOLECYSTITIS AND JAUNDICE (No Obstructive Factor Demonstrated)						
Number Cases	2	1	0	1	0	0
Proved	2	1	0	1	0	0
TOTAL CASES	75	48	14	7	4	2
PROVED	58	37	9	7	3	2

TABLE II

HEPATOCELLULAR JAUNDICE

		NEGATIVE		POSITIVE		
PARENCHYMATOUS (Toxic Hepatitis, Catarrhal Jaundice)		0	–	++	+++	++++
Number Cases	58	17	3	2	10	26
Proved	8	3	1	1	0	3
NEOARSPHENAMINE						
Number Cases	4	3	1	0	0	0
Proved	0	0	0	0	0	0
CIRRHOSIS						
Number Cases	17	4	1	5	2	5
Proved	8	2	0	2	1	3
TOTAL CASES	79	24	5	7	12	31
PROVED	16	5	1	3	1	6

DISCUSSION

In the cases of obstructive jaundice, the cephalin flocculation test showed an accuracy of 83 per cent. It would appear that the problem of differential diagnosis of "medical" vs. "surgical" jaundice was nearer a solution by this reaction. Unfortunately, as will be shown later, a fairly large number of instances of parenchymatous jaundice gave negative reactions. At any rate, our interpretation of the 1-plus reaction as negative appeared to be well grounded.

In cases of "infectious" hepatitis the reaction was positive in about two-thirds of the cases. Although the sensitivity of the test does not appear to be remarkable, its reliability when positive is great, for whenever a 3-plus or 4-plus reaction occurred, the diagnosis of parenchymatous liver disease was confirmed

by chemical or pathologic data. Only rarely did a case of obstructive jaundice give a positive reaction, and then only weakly positive such as 2 plus.

The type of toxic reaction to organic arsenical therapy described by Hanger and Gutman,[3] i.e., an intrahepatic obstructive icterus, was encountered in 4 cases. All were in individuals who received only two or three doses of the arsenical (in these instances, neoarsphenamine). The onset of icterus was sudden and in 2 cases the jaundice was prolonged. It should be noted that in Nadler and Butler's[6] cases as well, the flocculation test was negative in all instances.

In cirrhosis, the cases exhibiting evidence of active liver disease as evidenced by jaundice, as well as other stigmata of hepatic insufficiency yielded positive reactions in only half of the cases. In an additional three cases not tabulated because jaundice was absent, two were 0, and one was 1 plus.

SUMMARY AND CONCLUSIONS

1. By means of the cephalin flocculation test 164 assorted cases of jaundice were studied over a 22-month period, with interest directed toward differentiating obstructive from nonobstructive icterus.

2. Of 75 cases of obstructive jaundice, 62 were "negative."

3. Of 58 cases of hepatitis, 20 were negative and 38 were positive.

4. Of 4 cases of postarsenical icterus, all were negative.

5. Of 17 cases of cirrhosis with jaundice, 12 were positive.

6. In 3 cases of hemolytic jaundice, the reaction was negative in 2, positive in 1.

7. In our laboratory, the cephalin flocculation test reached its highest degree of accuracy in the obstructive jaundice group, but paradoxically was more reliable in the hepatitis group because false positives were a rarity.

8. To our mind the 1-plus reaction is of no significance in distinguishing obstructive from nonobstructive jaundice.

9. In general, the cephalin flocculation test may offer assistance in the differential diagnosis of jaundice. However, in our hands its accuracy did not concur with the results published by Hanger.

We wish to express our gratitude to the Difco Company of Detroit for their supplies of cephalin and to the Eli Lilly Company of Indianapolis for merthiolate.

REFERENCES

1. Hanger, F. M.: The Flocculation of Cephalin-Cholesterol Emulsions by Pathological Sera, Tr. A. Am. Physicians 53: 148, 1938.
2. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Investigation 18: 261, 1939.
3. Hanger, F. M., and Gutman, A. B.: Postarsphenamine Jaundice Apparently Due to Obstruction of Intrahepatic Biliary Tract, J. A. M. A. 115: 263, 1940.
4. Pohle, F. J., and Stewart, J. K.: The Cephalin-Cholesterol Flocculation Test as an Aid in the Diagnosis of Hepatic Diseases, J. Clin. Investigation 20: 241, 1941.
5. Rosenberg, D. H.: The Cephalin Cholesterol Flocculation Test in Cases of Disease of the Liver, Arch. Surg. 43: 231, 1941.
6. Nadler, S. B., and Butler, M. S.: The Cephalin Cholesterol Flocculation Test in the Jaundiced Patient, Surgery 11: 732, 1942.
7. Mateer, J. G., Baltz, J. I., Marion, D. F., Hollands, R. A., and Yagle, E. M.: A Comparative Evaluation of Newer Liver Function Tests, Am. J. Digest. Dis. 9: 13, 1942.
8. Mateer, J. G., Baltz, J. I., Marion, D. F., and MacMillan, J. M.: Liver Function Tests, J. A. M. A. 121: 723, 1943.

OBSERVATIONS ON THE INGESTION OF METHYL CELLULOSE AND ETHYL CELLULOSE BY RATS*

WILHELM DEICHMANN, PH.D., AND SYLVAN WITHERUP, B.S., CINCINNATI, OHIO

BECAUSE of the potential usefulness of methyl and ethyl cellulose as thickening and binding agents in the pharmaceutic and food industries, the possibility that they might have deleterious effects was investigated by feeding them to rats over a considerable period of time.

Methyl cellulose[1] is a white, fibrous, cellulose ether, soluble in cold water but insoluble in hot water and in most organic solvents. Heating causes most aqueous solutions of methyl cellulose to coagulate, but cooling and stirring restores them to their original smoothness and fluidity. Solutions of the compound are colorless, odorless, and tasteless. Ethyl cellulose,[2] also a cellulose ether, is insoluble in water but soluble in most organic solvents. Its solutions are colorless and odorless.

Acknowledgment is made to the Dow Chemical Company for the financial support of the investigation and for the supply of the materials in the form of Methocel (Lot M-368, viscosity 1500 centipoise) and Ethocel (Lot 2467, viscosity 20 centipoise).

GENERAL EXPERIMENTAL PROCEDURES

Young albino rats, male nad female, bred in this laboratory and all of approximately the same age and weight, were divided into 3 groups of 80, to be fed the two cellulose ethers and the control diet, respectively. They were caged in groups of twenty. The food was supplied in baskets of wire screening, and the drinking water, in bottles fitted with glass tubes, the quantities consumed being recorded weekly. Experiments were started in July and terminated in the following March, at which time a representative number of animals from each group was killed and examined for gross and microscopic tissue changes.

PREPARATION OF FOOD AND WATER

Eighty grams of methyl cellulose were wet with hot water, cooled to room temperature, and stirred until nearly smooth; then one liter of cold water was added, and after the mixture had been kept about twenty-four hours in the refrigerator, the whole was diluted to 1500 c.c. with water, mixed, and added to 10 kg. of Purina Fox Chow pellets. Thus 10 Gm. of the dried food contained 80 mg. of methyl cellulose (8,000 parts per million). The drinking water was prepared so as to contain 10 Gm. of methyl cellulose in one liter of water (10,000 parts per million).

Ethyl cellulose (120 Gm.) was dissolved in 3 liters of 95 per cent ethyl alcohol and added to 10 kg. of Purina Fox Chow. After the alcoholic solution had

*From the Kettering Laboratory of Applied Physiology, College of Medicine, University of Cincinnati.

Received for publication, August 19, 1943.

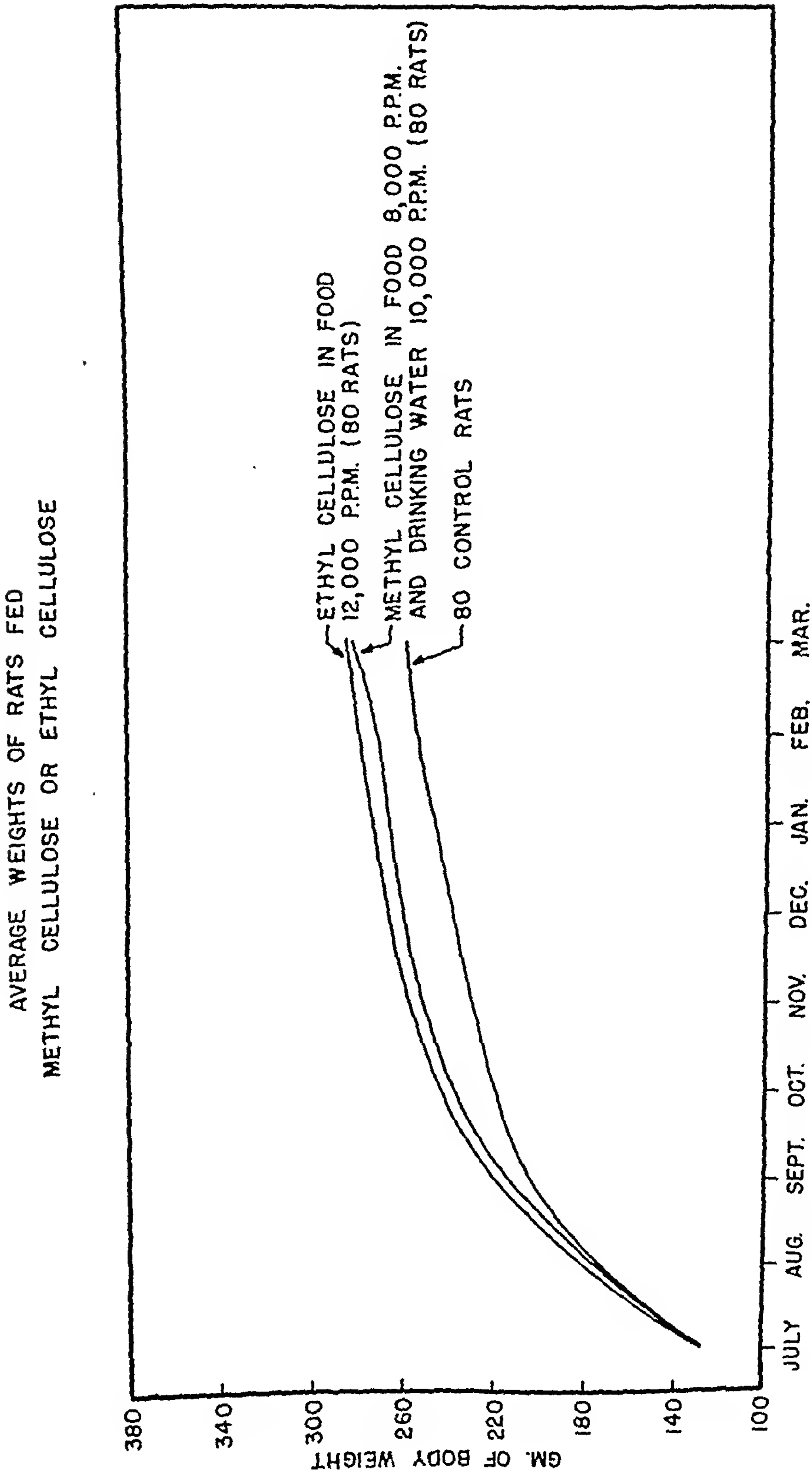

AVERAGE WEIGHTS OF RATS FED
METHYL CELLULOSE OR ETHYL CELLULOSE
ETHYL CELLULOSE IN FOOD
12,000 P.P.M. (80 RATS)
METHYL CELLULOSE IN FOOD 8,000 P.P.M.
AND DRINKING WATER 10,000 P.P.M. (80 RATS)
80 CONTROL RATS
GM. OF BODY WEIGHT
380
340
300
260
220
180
140
100
JULY
AUG.
SEPT.
OCT.
NOV.
DEC.
JAN.
FEB.
MAR.

Fig. 1.

soaked in, the pellets were heated for two days at 70° C. in order to expel the solvent. (Analyses proved that the alcohol had been expelled except for insignificant traces.) Ten grams of this food contained 120 mg. of ethyl cellulose (12,000 parts per million).

RESULTS

Group 1.—The 80 rats in this group were given both food and water containing methyl cellulose. The consumption of food varied from 13 to 19 Gm. per day, and the daily intake of water ranged from 25 to 34 c.c. so that the average total daily intake of methyl cellulose was about 436 mg. per rat, 125 mg. being ingested with the food and 311 mg. in the drinking water.

Group 2.—The 80 rats in this group ingested from 14 to 18 Gm. of food per day, the daily intake of ethyl cellulose being about 182 mg. per rat. These animals had free access to uncontaminated drinking water.

Group 3.—The consumption of food and water by the 80 control animals varied within the limits recorded in the case of Groups 1 and 2 above.

Fig. 1 portrays the weight changes that occurred in each group as a whole. The differences among the three groups in this respect were manifestly insignificant. No other evidences of differences in the behavior or well-being of the animals in the several groups were noted.

SUMMARY AND CONCLUSIONS

Eighty rats were given methyl cellulose in their food and drinking water over a period of eight months, and the same number were given ethyl cellulose in their food for a corresponding period. The daily ingestion of quantities of methyl cellulose amounting to about 0.44 Gm. per rat or of ethyl cellulose amounting to about 0.18 Gm. per rat produced no signs of illness. The consumption of food and water as well as the gain in weight by these animals was normal. No evidence was found of significant gross or microscopic abnormalities in the tissues of the animals. It is concluded that the feeding of methyl and ethyl cellulose in these concentrations is harmless to rats.

REFERENCES

1. "Methocel," a brochure published by the Dow Chemical Company, Midland, Michigan, 1942.
2. "Ethocel," a brochure published by the Dow Chemical Company, Midland, Michigan, 1940.

LABORATORY METHODS

GENERAL

AN INK-WRITING FINGER PLETHYSMOGRAPH*

HAROLD V. RICE, M.D., PH.D., WINNIPEG, CANADA

VARIOUS forms of finger plethysmograph are in use, employing photographic methods or using photoelectric cells. At least one form of ink-writing plethysmograph has been described,[1] though the data concerning its construction are not available. The present method has the advantage of being relatively inexpensive, has proved exceedingly simple and reliable to use, and can be made by anyone with a moderate knowledge of electronics. The advantage of an ink record of volume changes is obvious, since it makes possible observations over prolonged periods of time and provides a tracing which can be examined immediately, without the trouble and expense of developing it, as with the photographic method. The apparatus herein described also has the advantage that, since the plethysmograph chamber on the finger is connected to the recording unit only by fine flexible leads, slight movements of the hand do not tend to displace the plethysmograph relative to the finger. The plethysmograph is free in other words to move more or less as the hand moves.

The metal plethysmograph chamber (Fig. 1) is attached to a metal plate shaped to fit the palm and loosely bound to the palm by adhesive or spring clips at the edges. The latter method avoids obstruction of the venous return from the fingers. The distal end of the plethysmograph chamber is closed off with a slack condom rubber diaphragm. At the distal end of the chamber a bracket extends at right angles. This is made of metal, and being attached to the metal chamber is therefore at body or ground potential. Several parallel metal plates (4 to 8) are attached at right angles to this bracket. A moving arm is suspended on an insulator at the outer end of this bracket and is fitted also with parallel metal plates, which slide between the metal plates of the bracket without touching them, thus forming a variable condenser. The moving arm and its plates are made of aluminum foil (#35 gauge) for lightness, weighing only 4.0 Gm. Strength is obtained by flanging the edges where necessary. Movement is imparted to the movable arm by the condom diaphragm through a connecting pin. A gentle spring holds the pin constantly in contact with the diaphragm.

The finger is inserted into the plethysmograph with the "adjustment capsule" removed (cf. Fig. 1). An airtight seal is obtained simply by pressing

*Department of Physiology, University of Manitoba.
Received for publication, March 15, 1943.

vaseline between the edge of the plethysmograph and the skin. The "adjustment capsule" is then slipped onto the "stem." The adjustment screw is then tightened, thus depressing the thick rubber diaphragm beneath it, until the condom diaphragm is displaced outward to bring it into the mid-zone of its movement range. The two lead wires are then connected and the record may be taken. The preparations for a tracing take about a minute.

A calibrator is built into the plethysmograph. It consists simply of a thick rubber diaphragm, which is displaced by movement of a lever. It permits quick calibration of each tracing to be made. The calibrator must first be standardized against a known standard, the one in use having a displacement of .06 c.c.

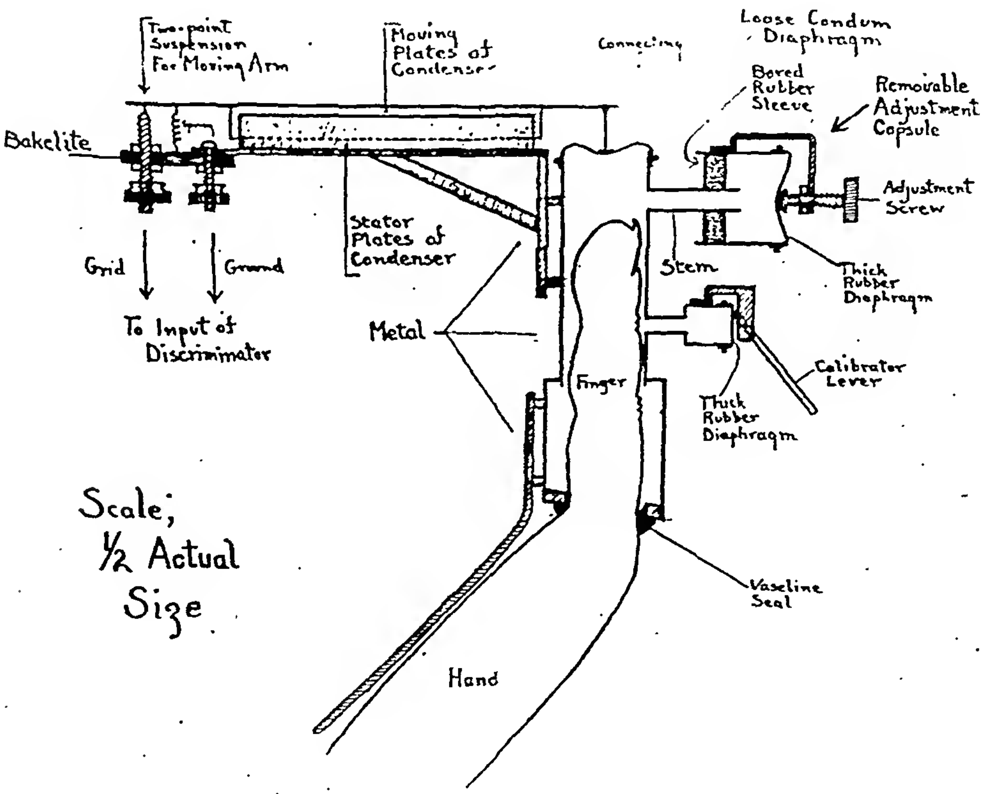
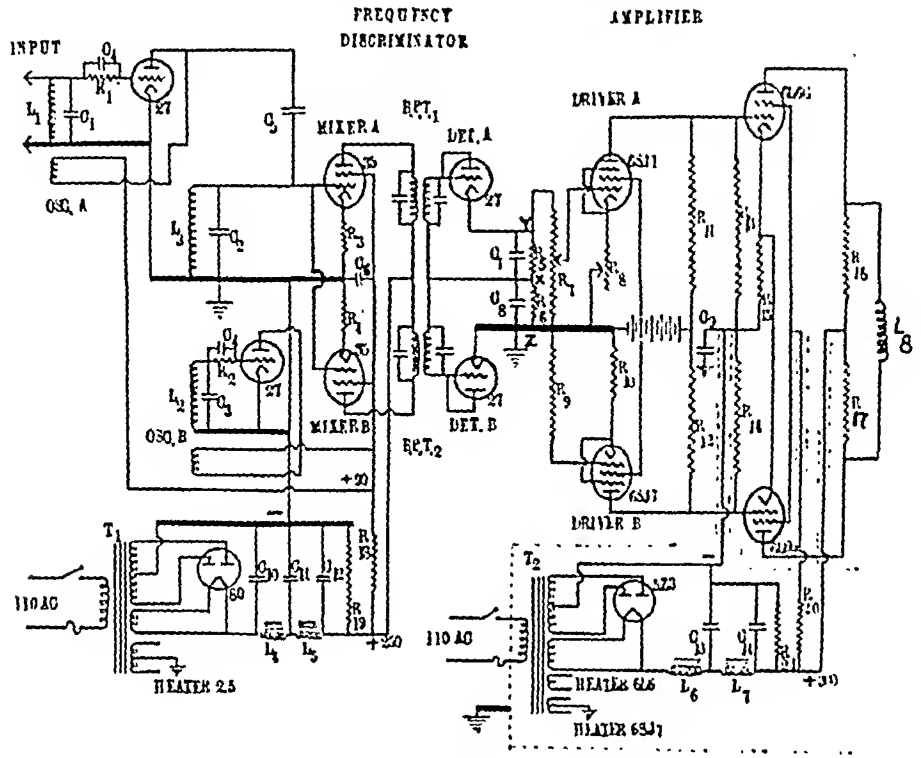


Fig. 1.—Scale diagram of finger plethysmograph.

The changes in finger volume are converted into changes of voltage which operate the recorder. The electrical circuit consists of a frequency discriminator which converts changes in frequency in an oscillator to voltage changes, which are amplified. Two oscillator circuits, A and B (Fig. 2), are tuned to beat against each other. The resulting beat frequency is fed into two mixer tubes in parallel. Mixer tube A passes its output into a band-pass transformer tuned to 175 kilocycles, and mixer tube B passes its output into a band-pass transformer tuned to 172.5 kilocycles. When the frequency of oscillator A is

tuned by its condenser C_1 to beat with oscillator B to produce a beat of 175 kilocycles, the maximum flow of current is produced through detector circuit A, and a maximum voltage drop is produced across resistor R_5. At this frequency the band-pass circuit B does not respond, and no current flows through detector circuit B. Consequently no potential difference is developed across



C_1, C_2, .0005 mfd variable. C_3, .00035 mfd mica. C_6, .5 mfd. C_9, 1 to 4 mfd.

C_4, C_5, C_7, C_8 .00025 mfd. C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, 8 mfd. electrolytic, 450 volts wtg.

R_1, R_2, 2 to 5 meg. R_3, R_4, 350 ohms. R_7, 500,000 ohms pot. R_8, 3000 ohms wire wound

R_5, R_6, R_{11}, R_{12}, R_{18}, 250,000 ohms. R_{10}, 1700 ohms. R_9, R_{13}, R_{14}, 500,000 ohms.

R_{15}, 240 ohms. R_{16}, R_{17}, 2000 ohms, 20 watts. R_{20}, 5000 ohms.

T_1, T_2, Power supply transformers.

B.P.T.$_1$, Band-pass transformer, 175 kc. B.P.T.$_2$, Band-pass transformer, 177.5 kc.

L_1, L_2, Tuning and oscillator coils, 540-1500 kc., wound on a common form.

L_3, Tuning coil. 540-1500 kc. L_4, L_5, 20 Henries, 100 ma. L_6, 10 Henries, 200 ma.

L_7, 1500 ohm field coil of recorder, 20 watts. L_8, Moving coil of recorder, 200 ohms.

Note; Power supply of output stage is all above ground and must be carefully shielded.

Fig. 2.—Schematic diagram of frequency discriminator and amplifier.

resistor R_6, so that points X and Z are at the same potential. Since there is a voltage drop across resistor R_5, point Y is positive with respect to point X and therefore also with respect to point Z. As a result the control grid of driver stage A becomes positive with respect to ground. As the potential of the control grid of driver stage B is not changed, the direct-coupled push-pull amplifier is thrown off balance, and moving coil L_8 connecting the two plates of the 6L6G output tubes is displaced. If oscillator A is tuned by its condenser to produce

a beat of 172.5 kilocycles, the band-pass circuit B responds to its maximum, and a maximum voltage drop occurs this time across R_6, making X negative to Z. Since no response is induced in band-pass circuit A at this frequency, no voltage drop occurs across R_5, and Y and X remain at the same potential. Y is thus negative with respect to Z, in consequence of which the control grid of driver A becomes negative with respect to ground, while the potential of control grid of driver B remains unchanged. The push-pull direct-coupled amplifier is put off balance in the opposite direction, and moving coil L_8 is moved in the opposite direction. When oscillator A is tuned to give a beat midway between 172.5 and 175 kilocycles, both band-pass circuits respond equally and an equal voltage drop occurs across R_5 and R_6. Y and Z are then at the same potential so that the control grid of driver A remains at ground potential, and the amplifier remains balanced. From this position of balance any change of frequency of oscillator A will change the beat frequency so that more current is induced in either band-pass circuit A or B.

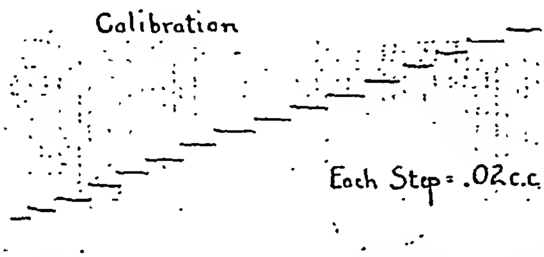


Fig. 3.—Record of successive increments of .02 c.c. into the plethysmograph from a pipette, showing the linearity of the response. Greater sensitivity can be obtained when desired.

When the plethysmograph is connected to the discriminator, the fixed plates of its variable condenser are connected to ground and the moving plates to the grid of oscillator A. By means of variable condenser C_1 the beat frequency is adjusted until the writing point connected with moving coil L_8 is brought to the mid-position. The correct setting of the condenser is easily found, for by turning it either way from the point of central adjustment, the writing lever should move an equal amount, first to one side, then to the other, as the movement of the condenser is reversed. Any variation of the position of the moving plates of the plethysmograph condenser, induced by changes of finger volume, causes a change in the frequency of oscillator A and therefore of the beat frequency and thus displaces the recording pointer.

In order to reduce undesirable interaction between the two oscillators, oscillator A is capacitatively coupled to the mixers and oscillator B is inductively coupled. The use of two mixers in parallel simplifies alignment of the band-pass circuits since they are then independent. Each circuit can be aligned separately without upsetting the alignment of the other. A separate high-voltage supply is used for the discriminator circuit. Condenser C_2 is used to tune the mixers to give the maximum response. The variable grid bias on only one of the 6SJ7 tubes, permits the voltage at the plates of the 6L6G tubes to be equalized. This is important, since with the direct coupling of the drivers to

the output tubes, slight inequalities in function of the driver tubes might cause serious imbalance in the output tubes. It must be noted also that the plate supply of the output stage is all above ground and requires special care in wiring and mounting. Since the heaters of the 6L6G tubes are also above ground, this heater winding is not center tapped. Condenser C_0 is to eliminate an A.C. component in the output. It should be a paper condenser to avoid leakage through it from the plate supply of the drivers which would tend to make the grids of the output tubes positive. The recording pointer is activated by a moving coil, suspended by springs in a dense magnetic field. The battery plate supply of the driver stage may be replaced by another power source. A potentiometer is used in the grid of the driver stage to control the gain of the amplifier. In order to vary the sensitivity of the variable condenser on the plethysmograph and to make it of service whether small or large changes of volume might occur, some of the plates of the stator can be removed in a moment from spring clips that hold them in place.

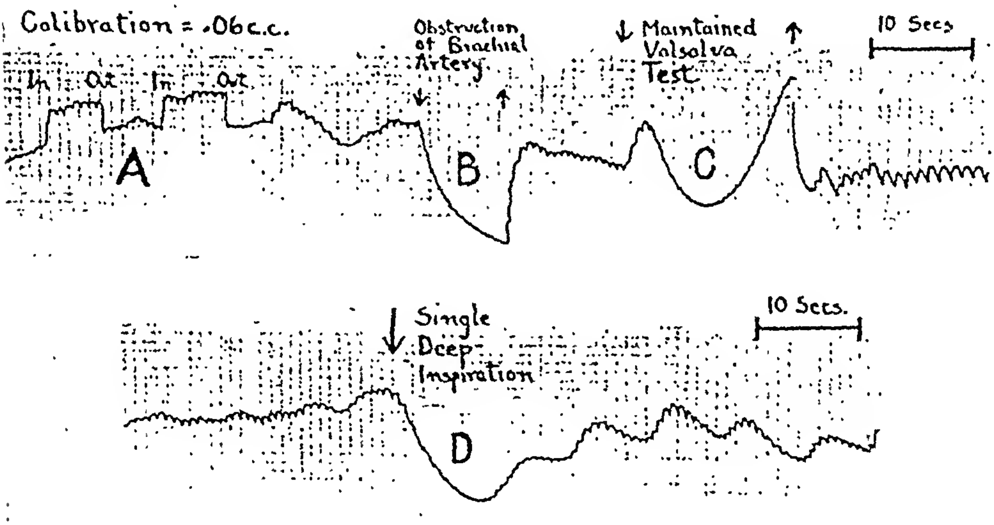


Fig. 4.—Tracings taken with the finger plethysmograph.

A, Calibration with .06 c.c. displacement, using the calibrator built into the plethysmograph.

B, Passive vasoconstriction due to obstruction (by pressure) of the brachial artery. Prompt return of volume on release of pressure.

C, Biphasic response with maintained forced expiration against the closed glottis. Prompt return of volume when forced expiration stopped, followed by large pulse waves.

D, Active vasoconstriction due to a single deep inspiration. Subsequent undulations due to respiratory movements.

Vasoconstriction or reduced finger volume causes downward movement of the recorder. Vasodilatation or increased finger volume causes upward movement of the recorder.

A calibration of the plethysmograph made by injecting successive volumes of 0.02 c.c. air into the chamber is shown in Fig. 3, indicating the linearity of the response.

It should be pointed out that the type of variable condenser on the plethysmograph does not lend itself to rapid volume changes. Frequency calibration curves have not been done, since the apparatus is designed primarily to record the slow volume changes of a finger due to changes of vascular control.

It is possible that some distortion would occur in the recording of the pulse curves, especially in the sharply rising phase. But such distortion, if present, would not lessen the reliability of records of the slow volume changes.

The actual apparatus in use was made from odd materials at hand. More recent types of tubes, etc., could profitably be substituted if available. Maximum voltage variations between Y and Z (Fig. 2) were about 20 volts. Maximum excursion of the pointer is about 6 cm. with a lever ratio of approximately 6 to 1.

Typical records of finger volume changes are shown in Fig. 4.

REFERENCE

1. Martin, S. J., Marcellus, F. S., and Sykowski, P.: Plethysmographic Studies With Special Reference to Waves of Respiration, J. Lab. & Clin. Med. 24: 111, 1938.

MACHINE-ROLLED STOPPERS FOR CULTURE TUBES

Harold W. Batchelor, Ph.D.,* Wooster, Ohio

Much of the loss of materials and of the labor required to make stoppers for culture tubes may be avoided if machine-rolled stoppers that can be used repeatedly are made by a lathe such as herein described.

The tongue of the stopper-spindle (1), two inches long, tapers from one-eighth of an inch at the shoulder to one-sixteenth of an inch at the tip. A second spindle for making hollow stoppers for use with pipettes is eleven sixty-fourths and ten sixty-fourths of an inch in diameter at the shoulder and tip respectively. The spindles, driven by a one-fortieth horsepower motor, are mounted on the motor shaft by means of a work arbor and chuck (2). A 20-gauge steel channel with flared flanges comprises the stopper forming table (3). The right half of the channel web, raised an amount equivalent to one thickness of the sheet steel, forms the tapered end of the stopper. A slot is cut in the left-hand flange to permit the spindle to function. The forming table is secured with two machine screws (4), near the left side, to an inclined forming table support (5). Two setscrews (not visible), mounted from below at the right side of the forming table support, bear on the under side of the forming table and permit tapering the smaller end of the stopper to any desired taper.

The stopper's diameter is controlled by an adjustable mechanism mounted on the upper end of the forming table support (5). The mechanism consists of a short channel (6), two setscrews (7), two flat springs (8 and 9), and a spring support (10). The lower setscrew (only partially visible) presses against the lower side of spring (8), one end of which is fixed to the lower side of the spring support (10). The angle of the latter conforms with the angle of the forming table support. The upper setscrew (7) presses against the upper side of a slightly bent flat spring (9), one end of which is free but whose

*Microbiologist, Ohio Agricultural Experiment Station.
Received for publication, February 6, 1943.

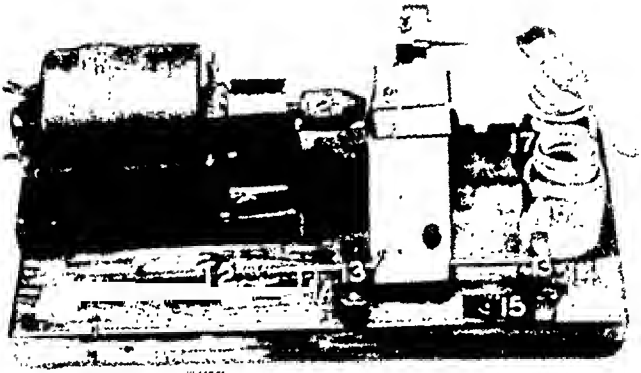


FIG.

FIG. 2

FIG. 3

other end is attached to the free end of spring (8). These two springs hold the forming table firmly against the spindle or cotton as the stopper is being rolled. Passing through, and tightly fitting into, the lower end of the forming table support runs a shaft (11), to whose left end is attached a coiled returner spring (12). Brass bearings mounted in walnut pillow blocks (13) support the shaft. Two adjusting studs (14) control the right and the left positions of the forming table support. A base, eight inches by sixteen inches, supports the entire mechanism.

The motor is started by means of the toggle switch (15), the spindle is moistened with water from (16), approximately the correct amount of cotton or other fibrous material is touched to the spindle, and the stopper is quickly rolled. The handle of the stopper may be painted or sprayed with a tinted plastic in solution (17), and the stopper may then be removed by pushing the forming table support to the right by means of post (18). Small stoppers may be made if a small-sized forming table is clamped to the right side of the larger forming table. Some stoppers made by the lathe are shown in Fig. 3. Stoppers have been made of absorbent and nonabsorbent cotton, fiber glass, and even steel wool. The latter two are only curiosities at the present time. The most satisfactory material yet used is a combustion-resisting DuPont cellulose acetate product known as 3-F Continuous Filament Batting. Being in an experimental stage, this material is not yet available commercially.

A NEW METHOD OF COUNTING PLASMODIA IN AVIAN MALARIA INFECTIONS*

HARRY BECKMAN, M.D.,† AND JANE SMITH, M.T.
MILWAUKEE, WIS.

THE best known and probably most used method of counting plasmodia in the peripheral blood in avian malaria infections is that in which the result of the count is recorded in terms of plasmodia per 10,000 erythrocytes. One of the forms of this "standard" method consists in counting the plasmodia seen in 1,000 erythrocytes and simply multiplying by 10; one counts erythrocytes up to 1,000 "in the head" while simultaneously recording plasmodia with pencil marks on a pad; or one counts the plasmodia on a pad or "in the head" and records erythrocytes on a mechanical counter; or one counts the erythrocytes first, notes on the verniers of the mechanical stage the limits within which the count was made, and then goes back and counts the plasmodia within these limits. In any case the resulting plasmodial count is multiplied by 10 and recorded as organisms per 10,000. We have not been able to satisfy ourselves that our counts are notably accurate when made in any of the above ways.

---

*From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee, Wis.
Received for publication, March 18, 1943.
†Partially supported by an Eli Lilly and Company grant.

A variant of the "standard" method consists in averaging the counts of 5 fields and dividing this number into 1,000 to determine the number of fields in which plasmodia must be counted in order to record the organisms per 10,000 (after multiplying by 10, of course); or one may divide directly into 10,000 and then make an actual count of the resultant very large number of fields. Working as we do, with a 92x objective and a 10x eyepiece, we find fields ideal for counting (i.e., well filled but with the erythrocytes not touching) to contain 90 to 100 cells; this would require us to see only 10 or 11 fields in order to obtain our 1,000 cells; we think this too few, but on the other hand to examine the 100 to 110 fields, which would be required to complete the count of 10,000, would certainly not be practicable. Another reason for our inability to be entirely satisfied with this method is that it assumes an almost perfect uniformity of smearing, which we are not certain that we invariably achieve.

The only other variant of the "standard" method with which we are familiar is to apply in each separate instance a formula by which to determine the number of erythrocytes to be examined, if the plasmodial count is to have an error of no more than 10 per cent. Gingrich[1] states this formula as follows: $N = 45.494 \frac{I-P}{P}$ in which N = the number of erythrocytes to be counted, P = the number of parasites per sample unit, and I = the sample unit (10,000 erythrocytes). At the present time, when a very large number of smears must be examined daily in all laboratories, we feel obliged to think of this method as having only academic interest.

Several years ago the senior author[2] offered a method which differed entirely from the "standard" method or any of its variants: A fixed number of plasmodia were counted, and then the count was recorded in terms of the time required to make it. This method, which enables one very simply to record degrees of infection as +, 2+, 3+, 4+, and 5+, served us in a practical way for a time, but it failed to prove sufficiently delicate to reflect slight differences in the antimalarial activities of the newer compounds which are being studied nowadays. We have therefore devised, and have been using with satisfaction for more than a year (i.e., in many hundreds of infections), the method now to be described.

DESCRIPTION OF OUR NEW METHOD

Using a 92x oil immersion objective and a 10x eyepiece, we give the slide a hasty examination to find an area in which we can range through a good many fields without encountering excessively wide variations in the thickness of the smear. Then the count is made:

The method consists in counting the number of organisms seen in three minutes of a *standardized type of search*. One starts the stop watch and then looks at every erythrocyte in the field. This is important; the whole field is not just quickly scanned and the number of plasmodia present noted, but instead every erythrocyte is looked at and the number of plasmodia seen during the course of this examination of the erythrocytes is counted. Then one turns to the next field and continues to examine erythrocytes and count plasmodia this way until the three minutes have elapsed. The total number of plasmodia counted in the three-minute period is then recorded as the index of the infec-

tion on that day. The watch is not stopped as one shifts from field to field, but it is stopped when glancing at it to determine the amount of elapsed time.

Two variants of the above are employed to make the method applicable to all the situations arising. (a) A negative slide is treated just as is a positive one; i.e., all erythrocytets are looked at in all the fields which are viewed in the three-minute period, but if a plasmodium is not encountered, the slide is simply recorded as negative. (b) If, in the examination of an apparently negative slide, a plasmodium is finally encountered before the three minutes have elapsed, the watch is stopped and reset at zero, and one starts over on a fresh count of three minutes beginning with this organism as plasmodium 1. This latter situation arises almost exclusively only on the first day of infections, for practically always on the days thereafter there is a sufficient abundance of organisms so that one is quickly able to find a field containing at least 1 plasmodium with which to begin the count.

TABLE I

COURSE OF THE *P. cathemerium 3H2* INFECTIONS IN FIVE CANARIES DURING THE FIRST FIVE DAYS AS COUNTED BY THE "STANDARD" METHOD AND BY OUR NEW METHOD

DATES	OUR METHOD			"STANDARD" METHOD		
	COUNT	TIME REQUIRED	R.B.C. SEEN	COUNT	TIME REQUIRED	R.B.C. SEEN
Bird #975						
12-7	2	3′45″	2,841	1	4′45″	1,000
12-8	25	3′	2,290	6	4′50″	1,000
12-9	132	3′	2,258	91	4′30″	1,000
12-10	263	3′	2,279	195	4′30″	1,000
12-11	220	3′	2,187	125	4′30″	1,000
Bird #974						
12-8	3	3′45″	2,838	1	4′25″	1,000
12-9	25	3′	2,283	7	4′30″	1,000
12-10	89	3′	2,255	40	4′30″	1,000
12-11	203	3′	2,314	135	4′25″	1,000
12-12	153	3′	2,243	120	4′30″	1,000
Bird #548						
12-16	6	3′36″	2,743	0	5′	1,000
12-17	23	3′	2,231	8	4′30″	1,000
12-18	88	3′	2,187	49	4′30″	1,000
12-19	196	3′	2,267	113	4′30″	1,000
12-20	154	3′	2,261	95	4′30″	1,000
Bird #522						
12-16	3	4′10″	3,134	1	5′	1,000
12-17	27	3′	2,265	10	4′55″	1,000
12-18	77	3′	2,266	33	5′	1,000
12-19	156	3′	2,351	74	5′	1,000
12-20	93	3′	2,257	61	4′45″	1,000
Bird #514						
12-14	3	3′25″	2,494	2	4′40″	1,000
12-15	32	3′	2,231	16	5′	1,000
12-16	131	3′	2,304	75	4′20″	1,000
12-17	203	3′	2,202	142	5′	1,000
12-18	95	3′	2,124	122	5′	1,000

COMPARISON OF METHODS

Table I presents the data from a study of 5 birds infected with our 3H2 strain of *Plasmodium cathemerium*, each bird being represented by a smear obtained at 9:30 A.M. on the first five days of its infection. The counting by our method was done on the dates as shown during a period when these birds

were being employed as untreated controls in a routine study. The counting by the "standard" method was done at a later time, thus increasing the validity of the comparison, since it is highly unlikely that the same fields were selected for study in both instances. It will be seen that in using our method the count on the first day always required more than three minutes, since some time was employed in finding the first organism; but the counts of the succeeding days required only the arbitrary three-minute period. In the "standard" counts the time required varied from four minutes and twenty seconds to five minutes.

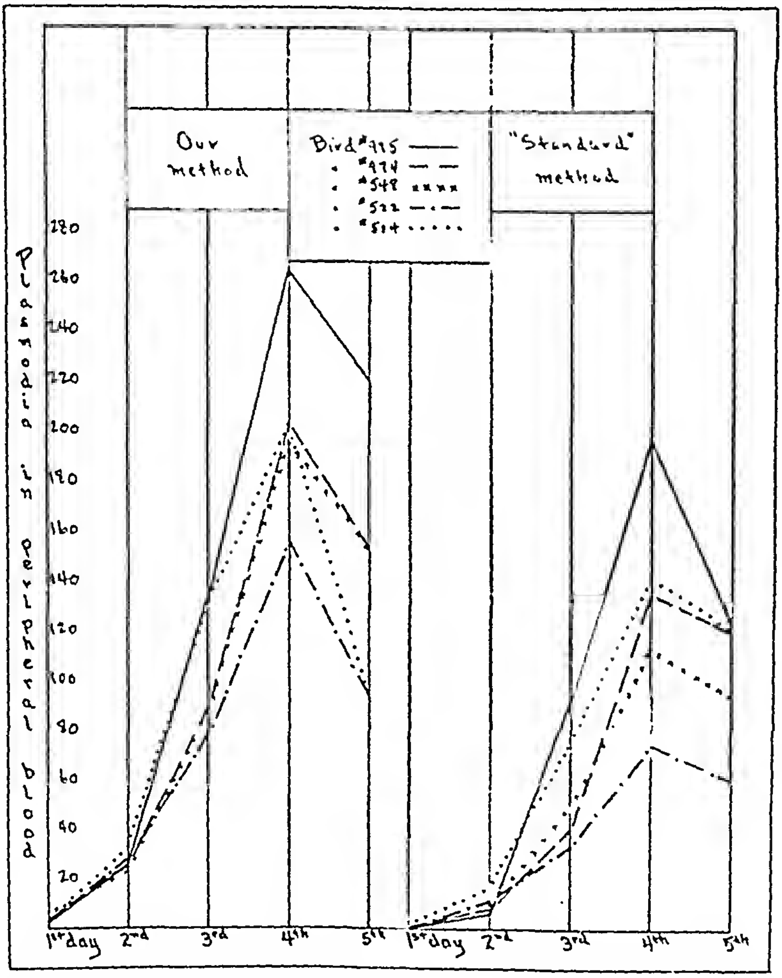


Chart 1.—Course of the *P. cathemerium* 3H2 infections in five canaries during the first five days as counted by the "standard" method and by our new method.

the average being four minutes and thirty-seven seconds. In the "standard" method 1,000 erythrocytes are examined; in our method more than twice this number are examined on all days after the first day; the extremes are 2,124 and 2,351, the average being 2,252. On the first day in our method we examine an average of 2,810 cells, or nearly three times as many as are examined in the "standard" method.

Chart I presents the counts in graphic form, the number of organisms being plotted as ordinates and the days of the infection as abscissae. The resemblance of the curves obtained by the two methods of counting seems to us in the main rather striking.

DISCUSSION OF OUR METHOD

We like our method for the following reasons: (1) No separate counts of erythrocytes and plasmodia are necessary; one merely gives the slide a hurried preliminary examination to select a suitable portion for study (a maneuver which precedes the use of all methods) and then begins examining erythrocytes and, almost incidentally, counting the plasmodia encountered. (2) There is astonishing uniformity in the number of erythrocytes examined, as shown in Table I, for the number of fields seen during the three minutes depends entirely upon the amount of time which must be devoted to the respective fields, more time being required of course to look separately at each erythrocyte in a crowded than in a scattered field. One quickly learns to omit fields in which only a few erythrocytes are present for this would obviously lead to error since the eye spends too much time in traversing unoccupied spaces, but this is easily accomplished by clicking off the stop watch and then clicking it on again when a more satisfactory area has been found. Then, too, no assumption of uniformity need be made with our method, since within reasonable limits satisfactory counts can be made, though the erythrocyte population of various fields differs quite widely. Each erythrocyte is looked at for the same length of time; that is the essence of the method. (3) By examining erythrocytes a full three minutes before declaring a smear negative we are reasonably sure it is really negative, for we have found upon innumerable occasions that a second study of three minutes and even a third practically never turns up a plasmodium. Furthermore, this three-minute study often enables us to find a plasmodium which would have been missed had we merely searched 1,000 erythrocytes; there is one such instance in Bird 548, as shown in Table I and Chart I. In our *P. cathemerium 3H2* infections the number of organisms increases sixfold to sevenfold in average instances between the first and second days; the necessity for detecting organisms on the very first day is therefore imperative if one wishes to begin treatment at the earliest possible moment.

As with all methods this has some disadvantages, but the only serious one of which we are as yet aware is that it can be employed only if one uses a stop watch having a mechanism to exclude periods of interruption and record only the actual time spent in examining erythrocytes rather than the total elapsed time. Nearly every laboratory, however, possesses one or more such watches. Of course a period of training is required to make one adept in the use of the method, but this is true of all precise procedures. We have found that for two workers to be able to check each other's counts requires only that they work together for a little while to adjust the amount of time actually spent in looking at each erythrocyte and also the speed of shifting from field to field.

SUMMARY

We have described a new method of counting plasmodia in avian malaria infections. Neither arbitrary numbers of erythrocytes nor plasmodia are

counted, but instead the slide is examined for an arbitrary period of three minutes, during which each erythrocyte is looked at individually and the plasmodial count is made rather incidentally to this examination of the erythrocytes. Despite the fact that one studies fields which vary considerably in density in using this method, the total number of erythrocytes examined varies only slightly from slide to slide. This method requires less time for its employment than the "standard" method, but enables one to examine more than twice as many erythrocytes. We believe the new method also permits earlier detection of patency than does the "standard" method.

REFERENCES

1. Gingrich, W.: Immunity to Superinfection and Cross-Immunity in Malarial Infections of Birds, J. Prev. Med. 6: 197, 1932.
2. Beckman, H.: An Experimentally Derived Method for Determining the Degree of Infection in Avian Malaria, Am. J. Trop. Med. 21: 151, 1941.

STREPTOCOCCAL HEMOLYSIS IN VARIOUS BLOOD MEDIA*

EUGENE F. TRAUT, M.D., AND MARY S. JOHNSON, B.S., CHICAGO, ILL.

CULTURES are commonly classified as hemolytic or nonhemolytic on the basis of their appearance as surface colonies on blood agar plates or slants. This classification becomes of considerable importance in view of the selective action of sulfanilamide preparations in therapy.

Most investigators have demanded of hemolytic strains of streptococci not only the production of hemolysis on an unspecified kind of blood plate but also more exacting criteria, usually the hemolysis of definite suspensions of red blood cells of specified origin. Other workers have encountered strains whose hemolyzing character depended upon the kind of blood used. This variability in hemolysis is often a source of confusion in the exchange of cultures between laboratories using blood from different sources. On moving our laboratory from the John McCormick Institute for Infectious Diseases, where sheep's blood had been used, to Rush Medical College where we used human blood, we noted that some stock cultures consistently nonhemolytic had become hemolytic. We investigated the constancy of this important change in cultural characteristics. We also applied to our strains the tests for hemolysis advocated by Brown.[1]

Cummings[2] favors inoculating suspensions of red cells to determine hemolysis. He regards as hemolytic only those streptococci that uniformly lake human, guinea pig, and mouse blood. Those strains producing hemolysis of only certain bloods he calls pseudohemolytic. Neither hemolytic nor "pseudohemolytic" strains change chocolate agar. He found "pseudohemolytic" streptococci regularly less virulent than the hemolytic.

---

*From the Laboratories of the Presbyterian Hospital and Rush Medical College.
Received for publication, March 10, 1943.

According to Brown[1] the green-producing streptococci and pneumococci produced less hemolysis in human blood agar than in horse or rabbit blood agar. The amount of hemolysis produced by B streptococci was insignificantly less on human than on horse or rabbit blood agar. Brown recognizes hemolysis only if produced within poured agar plates or in suspensions of red cells.

To hemolyze is usually regarded as a constant, unvarying characteristic. Strains have rarely been reported as losing or acquiring this property when identical conditions are reproduced.

Brown, Irons, and Nadler[3] noted a decrease in hemolyzing ability after repeated subculture. Dana and Murray[4] describe two strains of streptococcus pyogenes as hemolyzing horse blood but as producing green and not causing hemolysis in human blood. They found another strain producing green surrounded by a hemolytic zone in sheep and rabbit blood but typically hemolyzing all other bloods. They conclude that "since there are so few variations and since these may be due to some outside influence such as immune bodies in the blood, it would seem safe to assume that the hemolytic property of streptococci is fixed and constant."

Callow[5] says "the fact that a number of strains of nonhemolytic streptococci from the blood of rheumatic and nonrheumatic patients become hemolytic after prolonged cultivation may indicate that the hemolytic function can be suppressed."

METHODS

All blood plates were made by mixing 10 c.c. of fresh defibrinated blood with 100 c.c. of agar that had been melted and cooled to the proper temperature. The plates were incubated for 24 hours before being inoculated. Cultures were streaked out until individual colonies could be identified. These colonies were studied microscopically and macroscopically after 24 and 48 hours of incubation at 37.5° C. They were then re-examined after 24 and 48 hours' refrigeration. Five-tenths c.c. of broth culture of the same strains grown 24 hours in peptone ascitic broth was added to 1 c.c. of a 5 per cent suspension of washed sheep erythrocytes in normal saline. At the same time, a similar suspension of human cells was inoculated with these same strains. The supernatant fluid of these suspension cultures was examined for hemolysis after one hour in the refrigerator. The inoculations of a given strain on human and sheep blood were made on the same day, and the effects of colony growth were noted at identical times. Other observers concurred in our observations of the bacterial growths.

Because dextrose may inhibit hemolysis, it was not used in any of our media.

Of the 17 strains of streptococci studied, 12 were isolated from the rectum according to a previously described method.[6] Two were found in throats, one in the urine, one in the blood, and one was grown from the skin in erysipelas. Two strains of hemolyzing colon bacilli were obtained from the rectums of two patients. One strain of hemolyzing colon bacilli was obtained from a specimen of urine.

RESULTS

One strain of streptococci (Bo) consistently hemolyzed human blood while producing no hemolysis in sheep blood. This strain produced green on sheep blood on one subculture, and 2 months later was indifferent on the same medium. It never produced green on human blood. Two strains of streptococci (Oss, Endo) hemolyzed sheep blood but not human blood. Six strains of streptococci (Gen, Wym, Hal, Mil, Mon, erysipelas) hemolyzed cultures of both bloods but hemolyzed sheep blood more. One strain (Wym²) produced more hemolysis in human blood than in sheep blood. Nine strains, including the culture from scarlet fever and the 3 strains of hemolyzing colon bacilli, hemolyzed human and sheep blood typically and to the same extent.

SUMMARY

The ability or inability of several strains of streptococci to hemolyze depended partly or wholly upon the origin of the blood used to enrich the culture medium. Hemolyzing ability or lack of it remained constant at least four months for a given type of blood used in culturing. Besides an absolute difference between colonies hemolyzing one kind of blood and failing to hemolyze another, there were clear-cut quantitative differences between amounts of hemolysis produced in the two kinds of blood. The variety of blood that was to make up a medium to test hemolysis might be either sheep or human but was always the same for a given strain. Taken individually, there was a great regularity in the reaction of these strains as regards hemolysis in a given kind of blood. In the group of bacteria tested, human blood was as often hemolyzed as sheep's blood.

The hemolyzing or nonhemolyzing character of a given strain remained constant for a given type of blood regardless of the method used to test hemolysis.

REFERENCES

1. Brown, J. H.: The Use of Blood Agar for the Study of Streptococci, Monographs of the Rockefeller Institute for Medical Research, No. 9, Jan. 21, 1919.
2. Cummings, W. M.: On the Pseudohemolytic Streptococci Isolated From the Sputum in Pulmonary Tuberculosis, J. Path. & Bact. 20: 279, 1927.
3. Brown, E. V. L., Irons, E. E., and Nadler, W. H.: The Localization of Streptococci in the Eye, Arch. Ophth. 45: 229, 1916.
4. Dana, D. A., and Murray, F. J.: A Study of Hemolytic Properties of Streptococci on Various Blood Agars, J. Infect. Dis. 63: 122, 1938.
5. Callow, B. R.: Bacteriologic Investigation of the Blood in Rheumatic Fever, J. Infect. Dis. 52: 60, 1933.
6. Herrold, R. D., Traut, E. F.: A Simple Method of Making Cultures of the Rectum, J. Infect. Dis. 45: 197, 1929.

OBSERVATIONS BASED ON A LARGE SERIES OF COMPLEMENT TITRATIONS

MELVIN E. KOONS, M.Sc., M.P.H.*
GRAND FORKS, N. D.

DURING the past four or five years the successful application of the Kolmer complement fixation test in routine testing of blood specimens has been due, we believe, to the use of high titer complement. In recent conversations with various serologists who represented many laboratories, however, the author was much surprised to hear the many complaints regarding complement titration.

In the Public Health Laboratories of the North Dakota State Department of Health little trouble has been experienced with complement per se, when used in the Kolmer complement fixation test. In view of the difficulties exercised in the complement titration, it was deemed worthwhile to point out the experience in North Dakota. Two points bear out the above statement: (1) the consistently low titer of complement as used in other serologic laboratories, and (2) the available records of high titer complement as used in the North Dakota Public Health Laboratories.

From experience most serologists will agree that for the efficient performance of complement fixation tests, good complement is a decided asset. The use of poor complement invariably gives a "sloppy" run, accompanied by slow clearing of the test tubes with the end result of a large number of doubtful readings which are eventually proved to be negative.

Many authors have pointed out the importance of diet upon the quality of complement; however, no attempt will be made here to cite any specific literature. A complete picture of the entire system used in North Dakota should suffice, and it is hoped some benefit may be derived therefrom.

CARE OF GUINEA PIGS

The guinea pigs used as a source of complement are full-grown male or nonpregnant female animals purchased on the open market. No particular breed is especially purchased. The pigs are purchased when small and grown to maturity in our laboratories. The animals are quartered inside the year around, with the temperature of the room varying from 60° to 80° F., depending upon outside conditions. They are kept in metal cages of a size two by four feet, averaging from 10 to 12 per cage. Males and females are kept separately. The cages are cleaned once a day and animals are fed each morning.

The diet consists chiefly of Purina Rabbit Chow Checkers (entire ration) manufactured by the Ralston Purina Company, St. Louis, Mo. The ingredients are ground oats, corn meal, wheat germ, soy bean oil meal, corn germ meal,

*Director, Division of Laboratories, North Dakota State Department of Health.
Received for publication, March 22, 1943.

alfalfa meal, wheat middlings (gray), molasses, riboflavin supplement, 1.5 per cent calcium carbonate (limestone), 0.5 per cent iodized salt. A portion of these checkers is kept in front of the animals at all times. During the winter the pigs are fed freshly cut carrots at least once a day and during the summer freshly cut grass is substituted for the carrots. Hay is placed in the cages twice a week and year around. Fresh water is put in each cage every morning and twice a day in extremely hot weather. With this diet our experience has been that the pigs gain weight steadily and always look "well."

BLEEDING OF ANIMALS

All feed is removed from guinea pigs at least eighteen hours before they are bled, although water is allowed in the cages. Usually from 6 to 10 pigs are bled at each period. Approximately 8 c.c. of blood is collected from each animal. The pigs are bled about once every six weeks.

The animals are lightly anesthetized with ether and bled from the heart with a 20 gauge needle and a 10 c.c. syringe. The blood from each animal is placed in a separate test tube and allowed to remain at room temperature for approximately one hour. The tubes are then placed in the refrigerator overnight.

The following morning the serum is poured from the clots and centrifuged for ten minutes at approximately 1,500 revolutions per minute. The complement is pooled and placed in tubes containing about six c.c., then placed in the freezing compartment of an electric refrigerator at a temperature of approximately −8° C.

TITRATION OF COMPLEMENT

Complement prepared as above gives a high usable titer for approximately three weeks. The hemolysin used is of a high titer (generally 1:8,000) and is purchased from a commercial house. The antigen used is purchased directly from Dr. John A. Kolmer in Philadelphia.

The amount of complement needed for the day's run is thawed by placing tube in a small amount of cold water about thirty minutes before titration is to be set up. A 1:30 dilution of complement is used in the titration. All saline used in the titration is kept in the refrigerator in a large flask; hence cold saline is used throughout.

In a series of ten test tubes the complement titration is set up as shown in Protocol I.

It will be noted that after the complement antigen and saline are added the tubes are placed in water bath at 37° C. for only thirty minutes; after the addition of hemolysin and corpuscles, the titration is read when the tubes have been in the 37° C. water bath for only twenty minutes. As in all complement titrations, the smallest amount of complement just giving complete sparkling hemolysis is the exact unit. The next higher tube is the full unit which contains 0.05 c.c. more complement.

Table I shows the results obtained on 612 complement titrations by closely adhering to the above procedure.

Examination of Table I indicates clearly that most of the complement titrations show a titer of 0.25 c.c. or over. Of the 612 titrations, 84.8 per cent

gave a titer which could be used in the complement fixation test of 1:50 or above. This means that only 15.2 per cent of all complement titrations gave such titer that complement was used below 1:50. Further study shows that 11.0 per cent of those below 1:50 were usable at a dilution of 1:43. That the titers obtained were high is indicated by the fact that in 14.4 per cent of the titrations, complement was used at a dilution of 1:75 or higher. The largest number of titrations (70.4 per cent) showed that complement could be used at a dilution of 1:50 and 1:60.

PROTOCOL I

TUBE	COMPLEMENT, C.C. (1:30)	ANTIGEN DOSE, C.C.	SALINE SOLUTION, C.C.		HEMOLYSIN, C.C. (2 UNITS)	CORPUSCLES, C.C. (2 PER CENT)	
1	0.1	0.5	1.4	Water bath 37° C. for 30 min.	0.5	0.5	Water bath 37° C. for 20 min.
2	0.15	0.5	1.4		0.5	0.5	
3	0.2	0.5	1.3		0.5	0.5	
4	0.25	0.5	1.3		0.5	0.5	
5	0.3	0.5	1.2		0.5	0.5	
6	0.35	0.5	1.2		0.5	0.5	
7	0.4	0.5	1.1		0.5	0.5	
8	0.45	0.5	1.1		0.5	0.5	
9	0.5	0.5	1.0		0.5	0.5	
10	None	None	2.5		None	0.5	

TABLE I

RESULTS OF 612 COMPLEMENT* TITRATIONS
JANUARY 1, 1939 TO DECEMBER 31, 1942

NUMBER OF TITRATIONS	EXACT UNIT C.C.	FULL UNIT C.C.	PER CENT OF TOTAL NUMBER	TWO FULL UNITS DILUTION TO USE
17	0.1	0.15	2.8	1:100
71	0.15	0.2	11.6	1:75
201	0.2	0.25	32.8	1:60
230	0.25	0.3	37.6	1:50
67	0.3	0.35	11.0	1:43
16	0.35	0.4	2.6	1:37
7	0.4	0.45	1.1	1:33
3	0.45	0.5	0.5	1:30
	0.5	0.55		1:27
612			100.0	

*Frozen guinea pig serum used in all titrations (1 day to 3 weeks old).

The complement giving a titer of 0.15 c.c. and 0.1 c.c. were those titrated generally within three days after being obtained from guinea pigs. The opposite was found to be true, in that those titers on the lower end of the scale were generally encountered when complement was from 17 to 21 days old.

CONCLUSIONS

1. The successful application of the Kolmer complement fixation for syphilis in North Dakota has been due to a large extent to the use of high titer complement.

2. The care of guinea pigs, especially diet, is essential to the production of high titer complement.

3. Complement kept in a frozen state has proved to be highly satisfactory when not over three weeks old.

4. Scrupulous care as to technique will result in good complement titrations.

THE DERMOFLUOROMETER*

An Instrument for Objective Measurement of the Fluorescence of the Skin and Organs and the Objective Determination of Circulation Time and Capillary Permeability

Kurt Lange, M.D., and S. E. Krewer, New York, N. Y.

SOME time ago Lange and Boyd[1] showed that fluorescein, injected intravenously as a 5 per cent solution and irradiated by long wave ultraviolet can be seen in the capillaries of the skin and mucous membranes. This permits the determination of circulation time to different parts of the body and the establishment of adequacy of blood supply to these structures.[2, 3] Fluorescein can be watched traveling with the blood stream, permeating the capillary wall, and staining the tissue cells.

This method can be used routinely as a simple procedure to locate exactly the site of a vascular occlusion[2] or to ascertain how much blood flow to an organ or section of skin is diminished by the difference in depth of staining per unit of time. Extensive studies made it obvious that an awareness of an area of diminished blood supply was extremely valuable; quantitative measurement of the amount of fluorescein present at a given moment in any district of the skin or mucous membrane added considerable objective information.

Fluorescence of this dye is excited by blue and violet visible light as well as by an ultraviolet light source. This makes it possible to employ an incandescent lamp provided certain precautions are taken. While the ultraviolet light source equipped with adequate filters is unsurpassed for demonstration and visualization of vascular lesions, incandescent blue light is very convenient for actual measurements since it is smaller and produces less heat.

When we adopted the incandescent lamp for exciting fluorescence, it became feasible to design a compact unit which combines the light source as well as the phototube in rigid relative positions to each other (Fig. 1). The exciting (primary) blue light impinges upon the body while the yellow-green fluorescent (secondary) light (i.e., light of longer wave length than the primary light), of the blood stream, evoked by the blue light, is registered by the phototube; it is necessary to prevent the primary light from reaching the phototube by reflection from the skin or organ surface. This is accomplished by a filter F_1 (Fig. 2), in the primary light beam and filter F_2 set in front of the phototube. Filter F_1 transmits blue light only and absorbs practically all light of a wave length *longer* than 5000 Angstrom units. Conversely, filter F_2

*From the Department of Medicine, New York Medical College, Flower and Fifth Avenue Hospitals and Metropolitan Hospital-Service (Research Unit).

Aided by a grant from the John and Mary R. Markle Foundation.

Received for publication, April 8, 1943.

absorbs all light of a wave length *shorter* than 5000 Angstrom units and transmits mainly yellow-green light characteristic of the fluorescence of fluorescein. Since all these filters have a small undesirable leakage in the infrared, the phototube selected for this work is entirely insensitive to infrared radiation.

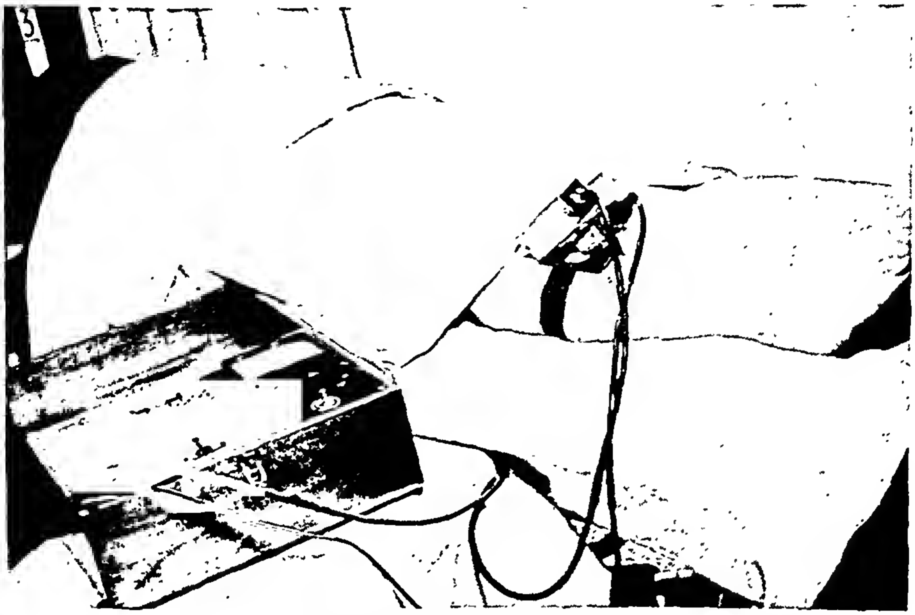


Fig. 1.—The dermofluorometer. The search unit comprising the light source and the phototube is attached to the leg of a patient.

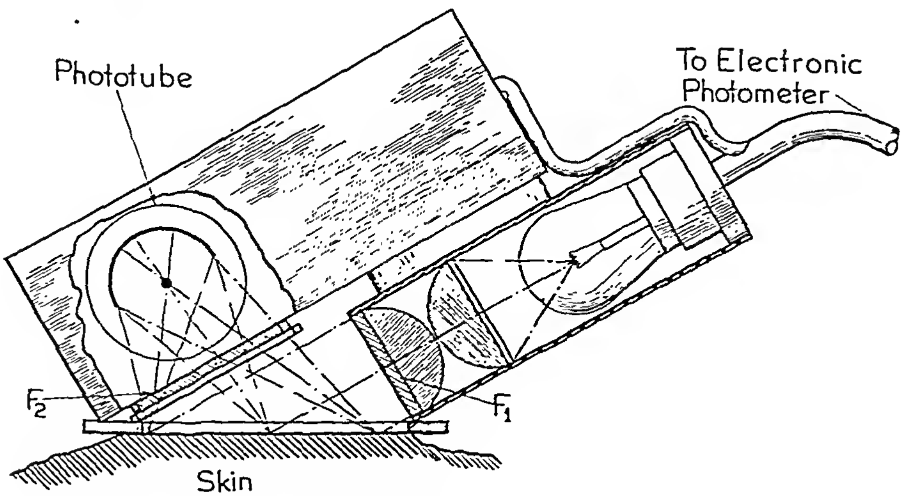


Fig. 2.—Schematic drawing of the search unit. F_1 blue filter in front of light source. F_2 yellow filter in front of phototube.

Because of these provisions, the light registered by the phototube before injection is negligible and is taken care of by a "background reading," which gives the actual degree of deflection of the instrument before the injection of fluorescein. The deflection following the injection of fluorescein therefore rep-

resents the effect of this injection quantitatively. The current output of the phototube is amplified approximately 3×10^5 in the electronic photometer.*

Table I shows the deflections of the instrument when standard solutions of fluorescein are tested; it proves that the device deflects proportionately to the concentration of fluorescein employed.

TABLE I

DILUTION OF FLUORESCEIN	DEFLECTION OF INSTRUMENT
1: 500,000	43.0
1: 1,000,000	22.0
1: 5,000,000	4.5
1:10,000,000	2.4

The instrument is used as follows: In a darkened room, several test spots of the body to be examined are touched with the instrument and the "background" deflection is noted. The instrument is then attached with a usual rubber strap to the spot at which the circulation time is to be observed. Ten cubic centimeters of a 5 per cent fluorescein solution are injected intravenously, and the time elapsing between the beginning of the injection and the initial deflection of the instrument is measured with a stop watch. This constitutes the circulation time to this area. A mouthpiece made from lucite, a translucent material, can be used to determine the circulation to the lips or gums if this is preferred to visual observation. Subsequently, all test spots are touched with the instrument, and the deflections of the photometer are read. From the results thus obtained, "background" readings are subtracted in order to obtain absolute fluorescein values. Thus a curve is obtained for each area of the body surface which indicates how much blood and, with it, fluorescein is reaching this district per time unit.

To prove that these deflections are really dependent upon blood supply, a blood pressure cuff was put around one thigh of a patient and inflated to a pressure between systolic and diastolic level. Subsequently comparative readings were taken on the normal and on the compressed leg of the patient, and the values were noted. Such a test is shown in Fig. 3. It indicates clearly that the values in the leg with partial occlusion are considerably lower than those in the normal leg.

To standardize the values found with different instruments, the following basis was adopted: one skin unit is the deflection caused by an alkaline fluorescein solution of 1:30,000,000 in a cuvette of 5 millimeters depth, the glass of which shows no absorption for the exciting light.

The first part of the curve thus obtained depends primarily upon the amount of blood reaching a given area per unit of time and upon capillary permeability. It will be shown in another paper that changes in plasma proteins within physiologic limits do not influence the amount of fluorescein available for diffusion into the tissue fluids. The last part of the curve is strongly influenced by the excretory function of the kidney.

Fig. 4 shows a typical normal curve recorded at the leg, and the curve of a patient with arteriosclerosis of the extremities, and one from a patient suf-

*The complete instrument was manufactured for us according to our specifications by the Photovolt Corporation, New York, N. Y.

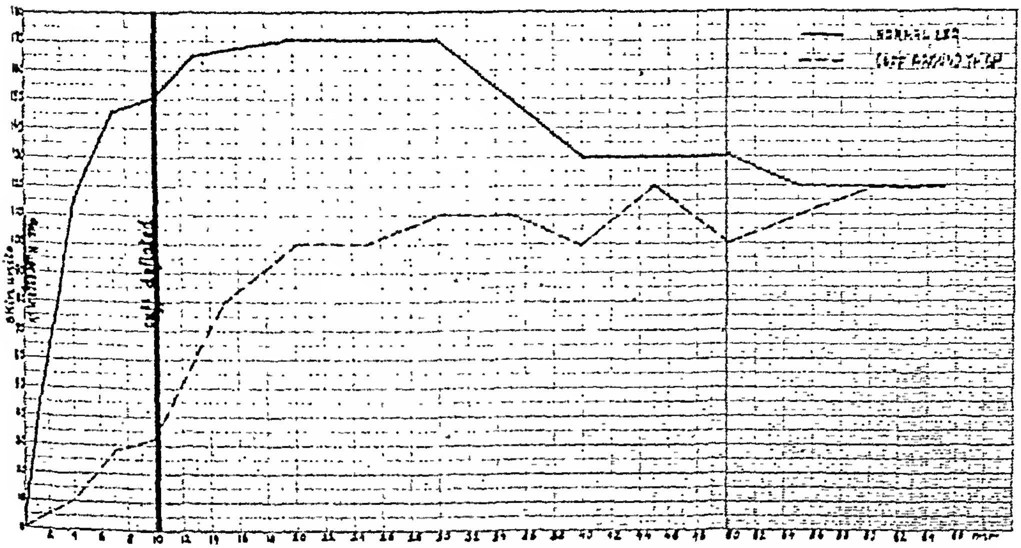


Fig. 3.—Dermofluorometer readings in a patient who had a blood pressure cuff inflated to a pressure between systolic and diastolic blood pressure around one thigh. 10 c.c. fluorescein were injected intravenously. Ten minutes after injection the pressure in the cuff was released.

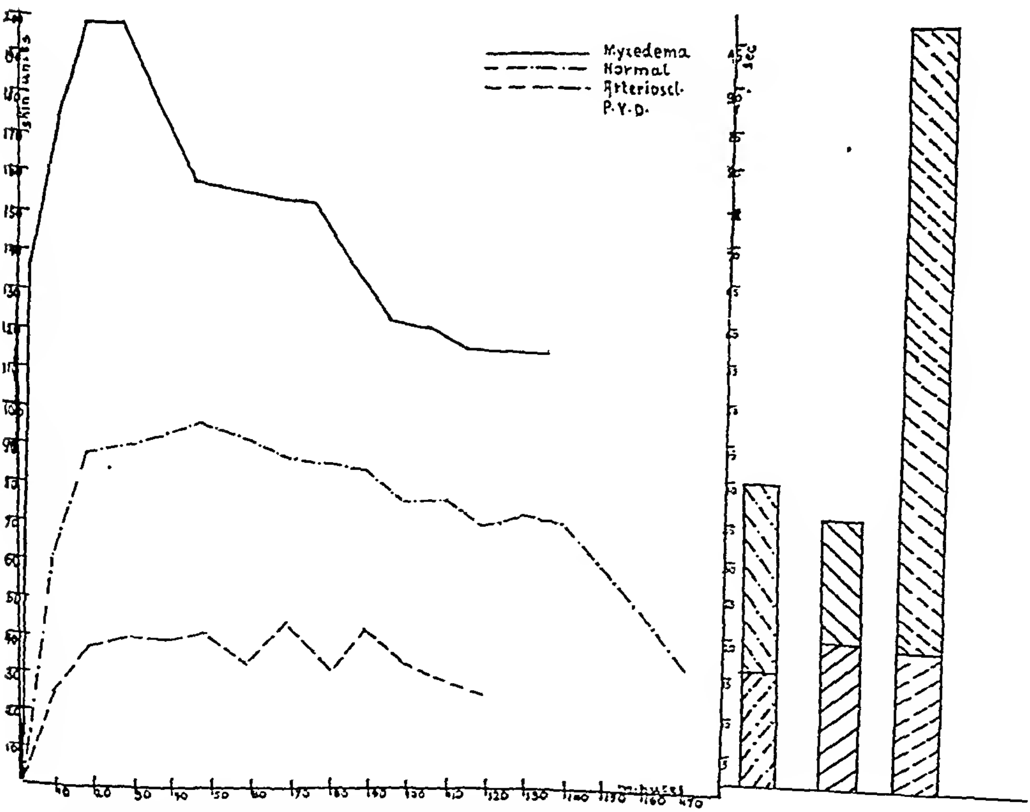


Fig. 4.—Dermofluorometer curves of three patients, one of whom is suffering from generalized arteriosclerosis, one from myxedema, and one, a normal person. The blocks give the circulation time in seconds from the arm to the lips and to the right leg respectively for each case.

fering from myxedema. In myxedema, the capillary permeability is especially high, and this may be the explanation for the peculiar skin condition and the tendency to form transudates, especially in the pericardium. By applying the instrument to different parts of the extremity of an individual with peripheral vascular disease, very often the exact place at which the artery narrows decidedly can be foretold. At the corresponding skin level, skin fluorescence suddenly decreases. At the same time the circulation time to a limb with a generalized arteriosclerosis is usually markedly prolonged. In a normal person the arm to leg circulation time should not exceed twice the arm to lip time. Formerly this could be seen with long wave ultraviolet, but now it can be measured exactly with the dermofluorometer.

SUMMARY

The instrument described permits quantitative measurements of the fluorescence values obtained when patients with vascular disorders are tested with fluorescein. A skin unit of fluorescein is defined. The exact location of a vascular stenosis can be determined with this device. Capillary permeability can also be determined.

REFERENCES

1. Lange, K., and Boyd, L. J.: The Use of Fluorescein to Determine the Adequacy of the Circulation, M. Clin. North America 26: 943, 1942.
2. Herrlin, J. O., Glasser, S. T., and Lange, K.: New Methods for Determining the Viability of Bowel, Arch. Surg. 45: 785, 1942.
3. Lange, K.: The Effect of Cold on Capillary Permeability, Bull. New York M. Coll. 5: 1942.

RAPID DIAGNOSIS OF MALARIA BY THE USE OF A WRATTEN LIGHT FILTER

RUBEN CARES, MAJOR, M.C., CAMP COOKE, CALIF.

CONTRAST filters are necessary in photography of colored or stained objects but can also be utilized in clinical microscopy. The visual contrast afforded can shorten the period of search required in a number of microscopic examinations before a negative report is issued.

A diagnosis of malaria after a short search for the parasite is not the rule. Many oil immersion fields must be examined before one intracellular organism is seen. When the young malarial forms, the "signet-ring" merozoites, are present, the closest study is required. In an early case or in a recurrence, the relative infrequency of infested red cells may cause the issuance of a false negative report. The paleness of the blue cytoplasm, often thinned out to little more than a line, allows an understandable oversight in running through hundreds of red cells. This is particularly true if no mature and larger forms are encountered, a not unusual condition.

Received for publication, April 15, 1943.

The thick drop method of crowding the red cells into the oil immersion field does not often disclose the intracellular location of the parasite. It is difficult to increase the staining intensity of the malarial organism by the routine hematologic stains.

A suitable light filter will produce the maximum contrast between the parasite and the red cells without of course affecting the stain for routine study. Blood stains are essentially a combination of methylene blue and eosin. It was found that of the various Wratten light filters* the E light red filter (series 23A) has a spectral transmission range parallel to that of eosin.[1] Moreover, this filter transmits a higher percentage of incident light in the red part of the spectrum than does eosin. Consequently the red cells actually appear lighter. The absorption of all blue (and green) light by the E filter renders the blue-stained elements distinctly darker. This contrasts sharply with the pale shadow or "ghost" rendition of the red cells. In a specified examination period 3 to 4 times as many parasites can be located as with normal lighting.

The simplest and least expensive form of the E filter is the dye-stained gelatin film supplied in one- or two-inch squares obtainable at photographic supply stores. A two-inch square can be cut up into a number of filters between cardboard mounts. These can easily be fitted to cover either the substage condenser or the ocular, and likewise can be instantly removed to study the located parasite by straight lighting.

Where analagous problems in microscopic studies exist, the enhanced contrast of light filters may similarly be utilized.

REFERENCE

1. Lukiesh: Handbook of Chemistry and Physics, ed. 26, Cleveland, Ohio, 1942, Chemical Rubber Publishing Co., p. 2129.

*Wratten light filters, Eastman Kodak Co., Rochester, N. Y.

MACROSCOPIC BLOOD TYPING*

A Modification of the Method of Thalhimer and Myron

S. W. Sappington, M.D., Philadelphia, Pa.

THE macroscopic method of blood typing, when compared with the standard microscopic method, offers advantages of economy in time and equipment and ease of performance and reading. It makes typing afield, such as outside of hospitals and under war conditions, simple and practical. If it can be shown to equal or exceed the microscopic method in accuracy, it would seem to be the method of choice. A gross method of typing has been described by Thalhimer and Myron[1] for use with the globulin fractions of pooled sera, the idea apparently being that the serum concentration thus attained would furnish agglutinating titers adequate for such a test. The method consists essentially in putting a large drop of A and B serum concentrate side by side on a card; placing a small drop of the undiluted blood to be tested at the side of each drop of serum; mixing the two drops with the adjacent blood; and, after a minute's rotation, observing the presence or absence of agglutination.

We used these concentrated sera and the method with the same satisfactory results indicated by Thalhimer and Myron, but while doing so and employing unpooled and unconcentrated sera for comparison, it was noted that single sera not infrequently gave equally good or superior agglutination. This suggested to us the possible value of an appraisal of unmodified single sera for the purpose. Accordingly, about 1,500 random sera were tested in various ways pertaining to the application of this method, controlling all results by the microscopic method. Sundry aspects of the results are submitted below.

Undiluted Versus Diluted Blood.—Undiluted blood taken directly from the finger necessitates an immediate performance and reading of the test but, on the other hand, requires little equipment and time and may be more desirable at the bedside and outside of hospitals. Blood diluted with an anticoagulant may be tested at one's convenience as to time and place and permits repetition of the test when desired. Our preference for diluted blood is based on the results of typing over 200 donors with both undiluted and diluted blood macroscopically and diluted blood microscopically. Here it was found that typing with undiluted blood was more open to error in reading results than with diluted blood, as the greater concentration in the former does not permit as clear a view of the developing agglutination. Likewise, it was noted that typing with anemic undiluted blood gave a clearer picture than normal concentrated blood for the same reason.

A blood dilution which gave a more readable agglutination than the use of pure blood and yet was not so diluted as to weaken the picture was one part

*From the Clinical Laboratories, Hahnemann Hospital. Technical assistance was given by K. E. Simmons and M. C. Krause.

Received for publication, April 22, 1943.

of blood to two parts of diluent. This dilution, while very satisfactory for macroscopic tests, is too low for microscopic work, which requires quite dilute suspensions.

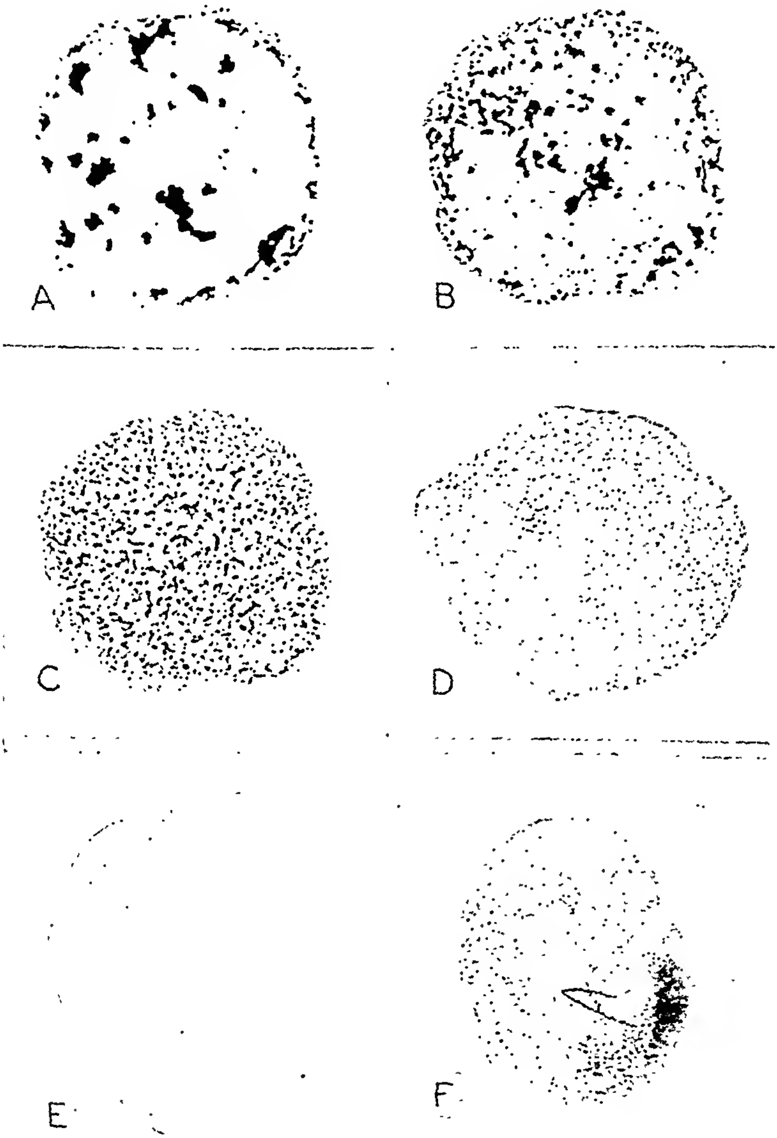


Fig. 1.—*A*, *B*, *C*, and *D* photographs show various degrees of macroscopic agglutination of red calls; *E* illustrates absence of agglutination; *F* photograph shows false clumping as seen occasionally in dried mounts.

The Blood Diluent.—Sachs,[2] in a study of the role of salt concentration in blood grouping tests, found that hemagglutination with unheated and heated serum was hastened and intensified in a high salt concentration, and he recommended that the cells for blood grouping be suspended in a 2 or 3 per cent sodium chloride solution. He stated that suspending the corpuscles in this stronger sodium chloride solution is a safe way of avoiding false negative reac-

tions such as may occur on cross matching. He also found that although the speed and strength of agglutinations are at first much increased with high salt concentration, the end titer may be lower.

On this basis, gross blood typing tests were done with a suspension of one part of blood in two parts of a 1 per cent solution of sodium citrate in 2 per cent sodium chloride. Comparison of the results with this solution with those attained with a 0.9 per cent saline solution confirmed the claims of Sachs. It was found that gross agglutination appeared uniformly more promptly in the 2 per cent salt suspension and that the advantage, while not great, was of sufficient value to warrant the use of the stronger solution.

Evaluation of Sera for Typing.—As a first preliminary test, an effort was made to type 100 unselected samples of blood serum by the gross method, and this was successful in all but 2 sera in which the titer was too low to give conclusive clumping. In a second preliminary test, an attempt was made to type 100 red cell suspensions using as typing sera A and B samples of the random sera of the first experiment. In doing this, sera of average or less than average agglutinating strength were purposely employed instead of the stronger sera. The entire 100 blood suspensions were thus typed correctly.

Having shown that blood may be readily typed by the macroscopic method using either known sera or known red cell suspensions, the problem was studied more exactly in a series of tests of donors' sera by noting carefully the time of gross agglutination and the size and character of the clumps and comparing these with the agglutinating titer of the respective serum. There were thus tested and titrated 100 A and 100 B sera. It was found that the isohemagglutinin titer and the agglutinative ability as measured macroscopically did not always agree quantitatively, and that in occasional individual cases, there was a marked discrepancy,* an experience similar to that of Thalhimer and Myron.[1] On the whole, however, as these authors point out in their work, there was a general trend toward correlation. A serum with a titer above 1/80 was usually adequate for gross typing. But for the purposes of the test, it was found better to rely on the gross results as seen in the rapidity and readability of the reaction than the titer. The rapid production of coarse clumps is the macroscopic evidence of maximum agglutination. Sera producing beginning agglutination in ten seconds or less and complete agglutination within

*In the titration tests, five dilutions of serum in normal saline were made as follows: 1:20, 1:40, 1:80, 1:160 and 1:320. To 1 c.c. quantities of such dilutions in small test tubes was added 1 drop of a 1:3 fresh suspensi[illegible]. This mixture was allowed to stand an hour at room temperature, [illegible] and then placed in the refrigerator overnight to be given a final re[illegible] first list below illustrates average comparative readings of titer and macroscopic clumping. The second illustrates selected examples of discrepancies in such readings: it is only fair to say that these are rare.

TYPE	TITER	GROSS CLUMPING	TYPE	TITER	GROSS CLUMPING
A	1:40	++	A	1:40	++++
A	1:320	++++	B	1:40	+++
A	1:80	++	B	1:160	++
A	1:160	+++	A	1:20	++
A	1:40	+	B	1:80	++
A	1:20	+			
B	1:80	+++			
B	1:40	++			
B	1:320	++++			
B	1:20	++			

one minute were graded ++++. Sera producing beginning agglutination in ten to fifteen seconds and complete agglutination in sixty to seventy seconds were graded +++. It is important to note that complete clumping may be present within these limits in the form of fine or medium-sized masses, though larger clumps may form later on standing. Sera grading 3 or 4 plus were serviceable for macroscopic typing. Thus measured, about 20 per cent of the A sera and 40 per cent of the B sera met these standards, so that one may anticipate that 1 out of 5 A sera and 2 out of 5 B sera will be adequate for macroscopic tests. It is especially important to use strong B sera on account of the subgroups of A bloods, and this is not difficult as strong B sera are quite common.

In the same way, fifty O sera were grossly tested and titrated against suspensions of A and B cells. Again it was found that the macroscopic clumping reaction afforded better indications of the agglutinative ability of the serum than titration. Usually O sera are stronger in anti-A agglutinins than anti-B agglutinins. In 50 sera, the anti-A were stronger than the anti-B agglutinins in 34 instances; in 15 instances, the anti-A and anti-B agglutinins were approximately equal in strength; and in one case, anti-B agglutinin was stronger. Of fifty O sera, 34 gave a 3 or 4 plus clumping reaction against A cells, and 12 gave a 3 plus clumping against B cells. As measured by macroscopic clumping, about 2 out of ten O sera may be expected to give a 3 plus or greater agglutination with both A and B suspensions. Such sera are adequate for control tests.

Cross typing of blood as a preliminary to blood transfusion involves the use of sera of unknown and perhaps low agglutinin values whether the tests are done by the macroscopic or microscopic method. In a series of 100 routine cross typings of donor and donee, in which both methods were applied, the gross method agreed with the microscopic test throughout. In our routine cross typing, however, we still employ both the gross and microscopic methods, as we wish by continued observation to be sure of the accuracy of the gross tests before dropping the standard microscopic one. Our further experience suggests this will be possible.

Reading of Results.—If the blood typing is done in the usual way, i.e., using A and B ++++ or +++ type sera against the donor's or recipient's red cell suspensions, the gross clumping is so prompt and self-evident in the great majority of instances that the type may be read macroscopically within a minute (Fig. 1 *A, B, C, D,* and *E*). If, on the other hand, an unknown serum is to be typed with A and B red cell suspensions, the rapidity and degree of agglutination are dependent on the unknown agglutinin titer, and may, in a serum poor in agglutinins, exhibit slow action and poor clumping, though it is nearly always possible to be certain of the type. Such results are quite exceptional, and doubtful cases, when controlled by typing through the blood suspensions, usually prove to be the type originally suspected.

With the usual large- and medium-sized clumps, the reading is perfectly simple. Infrequently, when the clumps are fine and formation slow, the reading is delayed and sometimes difficult. Here and in other instances, the additional use of a high titer O serum may be helpful. For example, if there is no

clumping with A or B serum and definite clumping with O serum, the results are erroneous. Again, when there is doubtful or unsatisfactory agglutination with A or B serum, the absence of clumping with O serum makes it fairly certain the type is O. If, after the use of O, A, and B sera, there is still doubt, the test should be reversed by trying the gross agglutinative effects of the serum of the blood to be tested against known emulsions of A and B cells. Under such circumstances, it should be kept in mind that if the serum to be typed is an O, it will usually clump A cells very promptly before there is any visible clumping in the B cells. It is therefore essential not to cease rotation of the card when A clumping appears, thus mistaking it for B serum, but to prolong it to be sure that the B cells also will not clump.

Final readings for type should be done while the mount is freshly wet. Card paper of the thickness recommended warps quickly under the influence of the blood-serum mixture, and correct reading is more difficult if the test is put aside to be read later. We do not feel it is worth while to preserve the dried individual tests as part of the clinical record, as the appearance changes somewhat in the dried specimen and in certain cases may give a false impression of type. In drying or dried blood-serum mixtures, a fine granulation may develop and be mistaken for true clumping by the inexperienced. (Fig. 1 *F*.) Negative bloods, that is, those like type O which show no clumping with A or B serum, are of necessity rotated longer in watching for possible agglutination; but this should not be continued too long, for example, four or five minutes, as a fine false clumping may be apparent.

One should not get the impression from the foregoing details, which are given largely as precautionary information, that macroscopic typing is a matter of hazards and difficulties. On the contrary, we found macroscopic typing quite as reliable as and more easily performed than the microscopic method. In the case of a type O patient showing weak irregular isoagglutinins which clumped not only her own cells but all other O types, the aberration was picked up quite as well by the gross as the microscopic method. And in the case of a sailor, typed by the Navy and wearing an O tag, the macroscopic method detected the error and showed he was an A type.

EQUIPMENT

A, B, and O ++++ or +++ typing sera in drop bottles. Keep, in intervals, in refrigerator.

White card index paper or No. 2 Bristol board cut in strips 10 × 4 cm.

Blood diluent, 1 per cent sodium citrate in 2 per cent sodium chloride.

Capillary pipettes and nipples. Small test tubes (7.5 × 1.3 cm.). Toothpicks.

Typing sera are easily obtained by taking an extra test tube of blood when bleeding donors. When ten or more of these sera are on hand, appraise them by the macroscopic method against A and B blood suspensions. Two or more out of ten will have a gross clumping value of +++ or ++++ and are serviceable for macroscopic tests.

METHOD

Place 8 drops of diluent, measured with a capillary pipette, in the small test tube, and add to it, collected with the same pipette, 4 drops of blood from

the patient's or donor's finger. This makes a 1 to 3 dilution which will keep; it is of the proper density and sufficient in quantity for repeated tests. Immediately or at one's convenience, place a large drop of each test serum, at least 7 mm. in diameter, on a labeled card strip, and beside it, a small drop of the diluted blood, one-fourth to one-third of the size of the serum drop. Both serum and blood drops will assume and retain an almost globular form on card paper. If two types of sera are used, the order from left to right should always be A and B; if three are used, the order should be A, B, and O. Mix each diluted blood and the respective serum with the flat end of a separate toothpick, spreading the mixture to a circle about 3 cm. in diameter. Avoid small circles. At once rock the card in various directions for a minute; let stand for twenty to thirty seconds; rerock the card for ten to twenty seconds. Read results while freshly wet. In rare instances, when clumping is slow or indistinct in one or both mixtures, further rotation may be necessary, but this should not be protracted beyond two or three minutes, as a fine false clumping may lead to incorrect readings. Dried mixtures not infrequently show this fine false granulation.

SUMMARY

In a study of the macroscopic method of blood typing described by Thalhimer and Myron, the following modifications are recommended: the use of diluted blood; a diluent of high salt concentration; the use of single, unconcentrated typing sera of high gross agglutinative ability. The reasons for these changes are discussed.

In our experience with about 1,500 controlled tests, the macroscopic blood method of blood typing here described equaled the microscopic method in accuracy and was superior to it in rapidity, simplicity, and economy. It has now been used as the routine method in our laboratories for four months with equally satisfactory results.

REFERENCES

1. Thalhimer, William, and Myron, S. A.: Globulin Fractions of A and B Agglutinating Serums for Blood Typing, J. A. M. A. 118: 370, 1942.
2. Sachs, Hans: Salt Concentration in Blood-Grouping Technique, Lancet 1: 473, 1942.

AN INHIBITION PHENOMENON IN PRECIPITATION TESTS FOR THE SERODIAGNOSIS OF SYPHILIS*

RACHEL BROWN, PH.D., ALBANY, N. Y.

THE purpose of this communication is to point out an inhibition phenomenon that may be a source of error in the precipitation test for the serodiagnosis of syphilis. The colloidal state of some sera is such that, although reaction between antigen and reagin apparently takes place, the formation of visible aggregates is inhibited. A serum of high activity may thus appear to be of only slight activity or to be nonreactive. The properties of the sera that give rise to this phenomenon are not understood; but the technique of precipitation tests should be such that sera of this character may be recognized and their reactivity interpreted correctly.

The inhibition phenomenon has been observed occasionally in an oversensitive procedure,[1a, 2] and there is reason to suspect that it may occur also in other tests. Although inhibition of precipitation may occur simultaneously with a prozone reaction,[3, 4] early recognized as a source of error, the two phenomena appear to be distinct and should not be confused. The properties of serum that inhibit precipitation do not disappear on dilution with physiologic saline solution. It has been found, however, that centrifugation† of the tests of such sera will cause the formation of large flaky aggregates. Similar treatment of typically negative or weakly reacting test mixtures does not cause precipitates of this character.

In Table I are presented the results of precipitation tests performed on four successive specimens of blood serum from a patient undergoing malarial therapy for syphilis of the central nervous system. The sera were tested undiluted and in a series of dilutions in physiologic saline solution with the antigen adjusted for oversensitive preliminary tests. With the first three specimens, reactions originally of insignificant degree became marked on centrifugation for five minutes at about 2500 revolutions per minute. The fourth specimen gave typical results. The attending physician knew of nothing in the patient's history to account for the change in serum properties.‡

These same specimens were examined with two other antigens for precipitating activity. When tested in a presumptive procedure,[5] the first three specimens failed to react, while the fourth specimen reacted as follows: undiluted, 2+; diluted: 1:5, 4+; 1:10, 4+; 1:20, 4+; 1:30, 4+; 1:40, 4+; 1:50, 4+; 1:60, 3+; 1:80, 2+; 1:100, +; and 1:150, –. They were also examined, for pur-

*Presented before the Eastern New York Branch, Society of American Bacteriologists, April 16, 1943, Troy, N. Y.

From the Division of Laboratories and Research, New York State Department of Health.

Received for publication, April 15, 1943.

†Centrifugation is a routine step in some precipitation procedures.

‡The sera from thirty-one other patients similarly treated did not show this peculiar property.

poses of comparison, with a carefully adjusted mixture of relatively pure lecithin and cardiolipin* sensitized with cholesterol. In this instance typical results were obtained by the regular procedure without centrifugation (Table I). Thus, although the colloidal condition of a serum is such as to inhibit the formation of a precipitate, this irregular behavior is also influenced by the antigen.

TABLE I

READINGS OF PRECIPITATION REACTIONS WITH SUCCESSIVE SPECIMENS OF BLOOD SERUM FROM ONE PATIENT

SERUM DILUTED WITH PHYSIOLOGIC SALINE	SPECIMEN NO. 167353			SPECIMEN NO. 170476			SPECIMEN NO. 172247			SPECIMEN NO. 175800		
	TEST WITH OVERSENSITIVE ANTIGEN		TEST WITH CARDIOLIPIN ANTIGEN	TEST WITH OVERSENSITIVE ANTIGEN		TEST WITH CARDIOLIPIN ANTIGEN	TEST WITH OVERSENSITIVE ANTIGEN		TEST WITH CARDIOLIPIN ANTIGEN	TEST WITH OVERSENSITIVE ANTIGEN		TEST WITH CARDIOLIPIN ANTIGEN
	REGULAR	CENTRIFUGED	REGULAR	REGULAR	CENTRIFUGED	REGULAR	REGULAR	CENTRIFUGED	REGULAR	REGULAR	CENTRIFUGED	REGULAR
Undiluted	–	2+	2+	–	2+	3+	–	2+	3+	+	Not	4+
1:2	–	2+	4+	–	2+	4+	+	3+	4+	3+	done	4+
1:4	±	3+	4+	±	3+	4+	2+	3+	4+	4+		3+
1:8	+	3+	3+	+	2+	3+	2+	3+	3+	4+		2+
1:16	+	2+	2+	±	2+	2+	+	3+	2+	4+		+
1:32	+	2+	+	–	2+	+	–	2+	–	3+		–
1:64	–	+	–	–	–	–	–	+		2+		–
1:128	–	–		–	–		–	–		+		
1:256	–	–					–	–		–		

Specimens were from a patient who had syphilis of the central nervous system and was undergoing malarial therapy; all had titers greater than 10 in the quantitative complement fixation test.[1b]

TABLE II

REPORTS OF PRECIPITATION TESTS WITH TWO SPECIMENS FROM THE WASHINGTON SEROLOGY CONFERENCE

		TEST			
SPECIMEN NO.	OVERSENSITIVE PROCEDURE	A	B	C	D
830	3+, +, ±, –* Positive Atypical	Positive	Negative	Doubtful	Negative
1090	±, ±, +, –** Doubtful Atypical	Negative	Positive	Positive	Positive

*The last three tubes containing 0.1 c.c. of undiluted serum and serum diluted 1:5 and 1:25 respectively with 0.02 c.c. of antigen, when centrifuged, read 4+, 4+, 3+.

**The first tube containing 0.05 c.c. of serum and 0.08 c.c. of antigen and the second and third tubes containing 0.1 c.c. of undiluted serum and of serum diluted 1:5 with 0.02 c.c. of antigen each, when centrifuged, read 4+, 4+, 4+.

These two specimens had titers greater than 10 in the quantitative complement fixation test.[1b]

Some of the discrepant results obtained in different test procedures may be due to this phenomenon. Table II records a few of the results obtained with two specimens, No. 830 and No. 1090, in the Washington Serology Conference.[2] Both specimens were from patients with diagnoses of syphilis of over four years' duration. Based on the results of the oversensitive procedure as performed routinely, the specimens were reported as "atypical positive" and "atypical

*The lecithin and cardiolipin were kindly supplied by Dr. Mary C. Pangborn.[6]

doubtful," respectively. Subsequently the tests were centrifuged, and evidence of marked reactions was obtained. The conflicting reports suggest that doubtful and negative results may be misinterpreted owing to failure to recognize the inhibition phenomenon.

The problem is one that should be considered for individual precipitation procedures; methods should be developed where necessary by which specimens that require special treatment may be detected. The recognition of the phenomenon in a given instance would require the use of more than one reaction mixture to indicate atypical findings.

CONCLUSIONS

The precipitation test for the serodiagnosis of syphilis is evidently subject to a phenomenon of inhibition in which the reaction between serum and antigen becomes manifest only under certain conditions. This inhibition appears to be distinct from the prozone phenomenon although the two may occur together. The phenomenon is of sufficient importance to require further investigation and careful consideration in the standardization of the technical procedure of precipitation to avoid the possibility of a false interpretation of the results.

REFERENCES

1. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research, New York State Department of Health, ed. 2, Baltimore, 1939, Williams and Wilkins, (a) p. 263, (b) p. 242.
2. Parran, Thomas, et al.: Preliminary Report on the Washington Serology Conference, Ven. Dis. Inform. 23: 161, 1942.
3. Kahn, R. L.: The Kahn Test. A Practical Guide, Baltimore, 1928, Williams and Wilkins, p. 16.
4. Brown, Rachel: Report on a Preliminary Precipitation Test as an Aid in the Serodiagnosis of Syphilis, J. Bact. 35: 337, 1938.
5. United States Public Health Service, Division of Venereal Diseases. The Serodiagnosis of Syphilis, Ven. Dis. Inform. (Supp. No. 9), 195, 1939.
6. Pangborn, Mary C.: Isolation and Purification of a Serologically Active Phospholipid From Beef Heart, J. Biol. Chem. 143: 247, 1942.

CHEMICAL

MODIFICATIONS IN METHODS FOR THE PRECIPITATION AND ASSAY OF INCREASED AMOUNTS OF PITUITARY GONADOTROPIC SUBSTANCES IN THE URINE*

PATRICIA H. SMITH, M.D., FULLER ALBRIGHT, M.D., AND ELIZABETH DODGE, A.B.
BOSTON, MASS.

THE demonstration of those pituitary gonadotropic substances in the urine which are follicle-stimulating has depended upon their precipitation and subsequent assay in a chosen test animal. Zondek (1930) precipitated urine with alcohol and used the ovarian response of immature rats as the end point of assay. Hamburger, in 1933, and Albright, Halsted, and Cloney, in 1935, used the alcohol precipitation method of Zondek, but observed the ovaries of infantile mice. Thomsen and Pedersen-Bjergaard (1936) used tannic acid and studied uterine enlargement together with ovarian weights in rats, and Callow and Callow (1937) modified their tannic acid method of precipitation. Levin and Tyndale (1936) also used tannic acid and were the first to use the weight of the infantile mouse uterus as the end point of assay. Catchpole, Greulich, and Sollenberger (1938) reported a method in which tungstic acid was the precipitating agent and in which mouse uterine weights were recorded. Heller and Heller (1939) used alcohol precipitation together with ovarian and uterine weights in immature rats as the test objects. In the experience of this laboratory all of the above procedures have been tried with the exception of the method in which tungstic acid was used, and no one procedure has proved entirely satisfactory. A method has therefore been developed which gives consistent and satisfactory results: alcohol was chosen as the precipitating agent; the volume used was increased, and enlargement of the uteri of infantile mice was the criterion of a positive test. Besides its use in the study of the menopause and in the sorting out of the various types of hypogonadism in males and females, it has led to the elucidation of two clinical conditions reported by Albright, Smith, and Fraser (1942) and Kleinfelter, Reifenstein, and Albright (1942).

CHOICE OF PRECIPITATING AGENT

Both alcohol and tannic acid as precipitating agents received extensive trial in this laboratory. The former has given excellent results; the latter as here

*The expense of this study was partly defrayed by the Committee for Research in the Problems of Sex of the National Research Council.
From the Biological Laboratory of the Massachusetts General Hospital and the Department of Medicine of Harvard Medical School.
Received for publication, August 5, 1943.

employed* was not consistently satisfactory (see Table I). At the suggestion of Fevold (1939), eight volumes of alcohol instead of four were employed, and the data showing the efficacy of alcohol as compared with tannic acid are given in Table I. Since certain patients after the menopause have so much pituitary gonadotropic substances in the urine that an assay can be made by injecting such urine without precipitation into animals, yields obtained by this procedure (so-called "direct method") were compared with those obtained by precipitation with alcohol or tannic acid followed by redilution.

Study of the table reveals that positive assays were obtained more frequently with the use of alcohol than with the use of tannic acid. Thus, of the ten cases studied, six showed better yields with alcohol than with tannic acid (Cases 1, 2, 3, 4, 9, and 10); in four cases (5, 6, 7, and 8) the yields were the same: in no instance was the yield with tannic acid better. The reason for the complete failure of the tannic acid method in certain instances (e.g., Cases 1 and 3) remains obscure. Since good results were obtained in some instances, it is conceivable that there is some error in technique in the method as used.

CHOICE OF METHOD OF BIOLOGIC ASSAY

In the selection of the method of biologic assay one may choose the rat or the mouse as the test animal and the ovary or the uterus as the significant organ of response. Hamburger (1933) pointed out that the mouse ovary was a more sensitive test object for pituitary gonadotropic substances than the rat ovary. Albright, et al. (1935) confirmed this. Levin and Tyndale (1937) reported data in the use of the uterine weights in immature mice as the criterion of a positive test but used tannic acid as the precipitating agent. This laboratory has followed the procedure of Levin and Tyndale in the use of the mouse uterine weight but has departed from their described procedure in that (a) alcohol was the precipitating agent, (b) different standards of normal uterine weights were established (videinfra), and (c) a different method of evaluating a positive test was used. Furthermore, it is strongly felt in this laboratory that quantitations of the active substances present should depend on response to various dilutions rather than on the degree of response of the test organ.

CRITERIA FOR NORMAL AND FOR ENLARGED MOUSE UTERI

To establish values for normal uterine weights in uninjected 21-day-old albino female mice weighing between 5 and 10 grams, 98 animals were autopsied and body and uterine weights recorded (Table II). The coefficient of correlation was 0.26 ± 0.06. This coefficient is more than four times its probable

*The method used was a modification of Callow and Callow (1937): A solution of analytical reagent tannic acid is freshly prepared just before use; 3 c.c. of a 10 per cent solution of tannic acid per 100 c.c. of urine are added to a concentrated first-morning specimen which has been filtered through Reeve Angel filter paper, No. 202, and pH adjusted to 4.5-5 before the addition of the tannic acid. The mixture is allowed to stand in the cold room 16 to 24 hours and the precipitate collected into 50 c.c. conical tu[illegible] The precipitate is washed with 5 c.c. of acetone per 100 c.c. of urine vol[illegible] placed in a suction desiccator overnight. The dry powder may be stored [illegible]ss of potency; 3 c.c. of borax buffer (50 c.c. of 0.2 M boric acid (61.8 Gm. [illegible] potassium chloride (74.6 Gm./L = 1.0 M) is mixed with 20.95 c.c. of 0.2 [illegible] and diluted to 200 c.c.) is added to the amount of dry powder obtained from 100 c.c. of urine, and 5 c.c. of distilled water is added. The mixture is stirred thoroughly, and 9 drops of phenol red added to the mixture are used as an indicator. Adjustment to pH of 8-9 is made by the addition of a 5 per cent solution of sodium carbonate, and the entire mixture is allowed to stand at room temperature for no less than one hour; 9 more drops of phenol red are added and the pH now adjusted to 7-7.5, using 2 per cent acetic acid. Distilled water is then added to make a final volume of 25 c.c. This gives a solution, each 2.5 c.c. of which is equal to 10 mouse units per 100 c.c. of the original urine.

TABLE I

RESULTS OF THREE METHODS OF ASSAY FOR PITUITARY GONADOTROPIC SUBSTANCES IN URINE

SOURCE OF MATERIAL	METHOD OF ASSAY					
	DIRECT		TANNIC ACID		ALCOHOL	
	M.U./100 C.C.	RESULT	M.U./100 C.C.	RESULT	M.U./100 C.C.	RESULT
Case 1. C. W. MGH 231528	40	+	20	Negative	20	+
	67	+	50	Negative	50	+
	100	+	70	Negative	70	Negative
Case 2. G. B. MGH 231481	40	Negative	10	+	10	+
	67	Negative	30	Negative	30	+
	100	Negative				
Case 3. E. R. MGH 32958	40	Negative	10	Negative	10	+
	67	Negative	30	Negative	30	+
	100	Negative	50	Negative	50	Negative
Case 4. A. S. MGH 17995	67	+	10	+	10	+
	100	+	30	+	30	+
	150	Negative	50	Negative	50	+
Case 5. A. S. MGH 17995	40	+	10	+	10	+
	67	+	30	+	30	+
	100	+	50	+	50	+
	150	Negative	70	+	70	+
			100	+	100	+
Case 6. A. M. MGH	40	+	30	+	30	+
	67	+	50	+	50	+
	100	+	70	Negative	70	Negative
	150	Negative	100	Negative	100	Negative
Case 7. H. K. MGH	40	+	10	+	10	+
	67	+	30	Negative	30	Negative
	100	+	50	Negative	50	Negative
			70	Negative	70	Negative
Case 8. A. S. MGH 17995	40	+	10	+	10	+
	67	+	30	+	30	+
	100	+	50	Negative	50	Negative
Case 9. E. R. MGH 32958	40	+	10	+	10	+
	67	+	30	Negative	30	+
	100	Negative	50	Negative	50	+
Case 10. Pooled spec., patient E. R. (MGH 32958) and patient A. S. (MGH 17995)	40	+	10	+	10	+
	67	+	30	Negative	30	+
	100	Negative	50	Negative	50	+

TABLE II

DATA SHOWING BODY AND UTERINE WEIGHTS IN 21-DAY-OLD FEMALE ALBINO MICE WEIGHING BETWEEN 5 AND 10 GRAMS

WT. OF ANIMAL GM.	UTERINE WEIGHTS MG.					TOTAL NO. ANIMALS	AV. UTERINE WEIGHT MG.
	2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9		
5.0-5.9	4	5	2			11	3.27
6.0-6.9	6	10	2	2		20	3.45
7.0-7.9	4	11	6	3		22	4.13
8.0-8.9	3	16	7	4	1	31	3.93
9.0-9.9	3	5	4	2		14	3.80

error and is therefore significant, although the significance is of slight degree. If the data in the group of animals weighing less than 7 grams are excluded, the correlation between the weight of the animal and the uterine weight is insignificant, so that if animals within the weight range of 7 to 10 grams are used, it is unnecessary to apply any correction factor to the uterine weights of in-

jected animals. To establish the fact that the injection of fluid alone had no effect on uterine weights, four normal mice received three times the volume of fluid used in the test in the form of normal saline. No uterine enlargement was noted.

In the series of normal control animals the average uterine weight (Table II) was 3.7 ± 0.94 mg. The uterine weight can fluctuate by chance alone within the limits of twice the standard deviation, or from 1.7 mg. to 5.5 mg. Even though the variation just given would cover 95 per cent of possible normal weights in those animals whose body weights lie between 5 and 10 grams, it was felt that a higher value, e.g., 6 mg., for the upper limit of normal would add significance to a positive result in a routine laboratory procedure. Therefore, a uterine weight of 6 mg. has been regarded as of beginning significance. The exact method of determining a positive test is presented.

PROCEDURE*

Precipitation.—Sixty c.c. of a concentrated first morning specimen of urine is filtered into a 1-litre Erlenmeyer flask. No specimen is accepted unless the specific gravity is at least 1.010. The pH is adjusted to 4.5-5, using nitrazene paper as the indicator and 75 per cent acetic acid as the acidifying agent. Eight volumes of cold alcohol which has stood at a temperature of not more than 24° C. are added. The mixture is rotated thoroughly and allowed to stand at this temperature for twelve to forty-eight hours. The active principle, which is water-soluble, settles to the bottom as a precipitate. The flask may remain at the reduced temperature for as long as a week without altering the results of the subsequent assay. The supernatant fluid is removed by suction (or by decanting if it has stood for the longer period of time), leaving a small amount behind in order to rinse out the precipitate into a 50 c.c. Pyrex conical centrifuge tube. All of the precipitate is collected into the conical centrifuge tube by use of a rubber policeman and repeated rinsings of the Erlenmeyer flask with supernatant fluid. The precipitate remains in the centrifuge tube throughout the subsequent steps of the procedure. After complete collection, it is washed twice with absolute alcohol, once with anhydrous ether, and placed in a desiccator overnight. There result a dry powder which can be stored indefinitely without loss of potency if kept dry. The active principle is prepared for assay by dissolving the powder in 15 c.c. of distilled water and adjusting the pH to 4.5-5 (as tested with nitrazene paper) using 75 per cent acetic acid. The water is allowed to stand in contact with the powder four to eight hours, and the mixture is stirred well at intervals no less than four times. If an insoluble residue remains, the clear supernatant fluid is used for injection. There is no loss of potency if this solution stands for several days, but loss of potency can occur, however, if the solution is allowed to stand as long as fifteen days at a temperature of 24° C. or less.

Whether complete precipitation is obtained even with alcohol is, of course, a debatable point. Comparison of the assays in which alcohol was used with the assays in which the direct method was used (Table I) show some discrepancy. One explanation may be incomplete precipitation; another may be that there

*The procedure here given has been slightly modified in the hands of Kleinfelter, Albright, and Griswold (1943) in that calculations are made in mouse units per twenty-four hours rather than mouse units per 100 c.c.

are substances in the unprecipitated urine which have a direct effect on the mouse uterus causing enlargement and which are removed in the various steps of the precipitating procedure. The discrepancy needs further elucidation.

Assay.—The assay is carried out on three 21-day-old mice, weighing between 7 and 10 grams. Each animal receives a total of 2.5 c.c. of the test solution prepared according to the procedure just described. Each mouse is injected as follows: 0.5 c.c. morning and afternoon of day 1; 0.5 c.c. morning and afternoon of day 2; 0.5 c.c. morning of day 3. Autopsy is performed the morning of day 4. Macroscopic enlargement of the uteri is considered a positive test. In doubtful cases the uteri are carefully dissected, pressed between filter paper to remove fluid, and weighed. The test is considered positive when the weight of the uterus is greater than 6 mg.

If only two of the three animals have uteri weighing between 6 and 7 mg., the test is not positive: but, if all three animals have uteri weighing between 6 and 7 mg., the test is considered positive. If one of three animals has a uterine weight of more than 7 mg. and the other two animals have uteri weighing less than 6 mg., the result of the assay is considered doubtful and is repeated not later than the next day. A conclusive result is usually obtained. When, according to this technique, three animals each receive 2.5 c.c. of the test solution and show no uterine enlargement, there is less than 1 mouse unit of pituitary gonadotropic substances present in 10 c.c. of the first morning specimen. When the mouse uterus is enlarged in animals which have received 2.5 c.c. of the test solution, the urine is known to contain a minimum of 1 m.u./10 c.c., or 10 m.u./100 c.c. In order to determine whether the gonadotropic content of the test solution is higher than 10 m.u./100 c.c., aliquots of this 15 c.c. of test solution are diluted from 2 to 9 times.* The assay is made with 2.5 c.c. of the diluted aliquots in the same manner as that described for the assay of the test solution.

DISCUSSION

Since the qualitative presence of pituitary gonadotropic substances can be demonstrated in instances where their concentration is high by injecting the urine directly into the test animals without preliminary precipitation, the point may well be raised whether testing for various levels is necessary. The injection of unprecipitated urine into the test animal gives a positive result only if the level is as high as 40 m.u./100 c.c. By alcohol precipitation the same volume of test solution injected is equivalent to 10 c.c. of the unprecipitated urine and, therefore, levels as low as 10 m.u./100 c.c. may be detected. For still lower values a modification of the Heller and Heller method is now used in this laboratory (Kleinfelter, Albright, and Griswold, 1943).

Using the method of concentration and assay just described, there was less than 10 m.u./100 c.c. in the urine of normal women throughout the menstrual

*In the use of three test animals a convenient table for making dilutions follows:

DESIRED ASSAY, M.U./100 C.C.	VOL. OF TEST SOLUTION NEEDED—C.C.	VOL. WATER TO BE ADDED—C.C.
30	3	6
50	2	8
70	2	12
80	2	14
100	1	9

cycle with the exception of one or two days at or near the time of ovulation. In menopausal patients any first morning specimen of urine was positive for a minimum of 10 m.u./100 c.c., and values as high as 100 to 150 m.u./100 c.c. were recorded in a few instances.

While enlargement of the uterus was the criterion of a positive test, it may be of interest that the ovarian response at the end of the 72-hour test period consisted of follicle enlargement only and corpora lutea were never seen. In addition, the vagina of test animals was potent in instances in which the level of the pituitary gonadotropic substance was of the order of 30 m.u./100 c.c. and over.

In the practical consideration of the test described, the greatest difficulty may be in obtaining animals which meet the specifications of the test. The procedure has merit in that the precipitate may be stored until such time as animals are available, and since the mortality rate of injected test animals is low, a minimum number of mice is needed.

SUMMARY AND CONCLUSIONS

1. In the method for the assay of increased amounts of pituitary gonadotropic substances in the urine the precipitation of those substances with alcohol is superior to precipitation with tannic acid.

2. Eight volumes of alcohol is probably better than four.

3. As a test animal, the mouse is more sensitive than the rat, and as a test organ, the uterus is preferable to the ovary.

4. The method used in this laboratory is presented.

The authors wish to thank Dr. Jacob Lerman for his help in the statistical evaluation of data, Dr. Hirsch W. Sulkowitch for his suggestions in the development of the method, and Dr. Rucker Cleveland for assistance in preparation of the manuscript.

REFERENCES

1. Albright, F., Halsted, J. A., and Cloney, E.: Studies on Ovarian Dysfunction. I. The Hormonal "Measuring Sticks" Available for Clinical Use and Values Obtained on Normal Individuals, New England J. Med. 212: 192, 1935.
2. Albright, F., Smith, P. H., and Fraser, R.: A Syndrome Characterized by Primary Ovarian Insufficiency and Decreased Stature Report of 11 Cases With a Digression on Hormonal Control of Axillary and Pubic Hair, Am. J. M. Sc. 204: 625, 1942.
3. Callow, R. K., and Callow, N. H.: Personal Communication, 1937.
4. Catchpole, H. R., Greulich, W. W., and Sollenberger, R. T.: Urinary Excretion of Follicle Stimulating Hormone in Young and Adolescent Boys, Am. J. Physiol. 123: 32, 1938.
5. Fevold, H. L.: Personal Communication, 1939.
6. Hamburger, C.: Studies on Gonadotropic Hormones from Hypophysis and Chorionic Tissue with Special Reference to Their Differences, Acta. path. et microbiol. Scandinav. supp. 17, 1, 1933.
7. Heller, C. G., and Heller, E. J.: Gonadotropic Hormone: Urine Assays of Normally Cycling, Menopausal, Castrated, and Estrin Treated Females, J. Clin. Investigation 18: 171, 1939.
8. Kleinfelter, H. F., Jr., Albright, F., and Griswold, G.: J. Clin. Endocrinol. In Press.
9. Kleinfelter, H. F., Jr., Reifenstein, E. C., Jr., and Albright, F.: Syndrome Characterized by Gynecomastia, Aspermatogenesis With A-Leydigism and Increased Excretion of Follicle-Stimulating Hormone, J. Clin. Endocrinol. 2: 615, 1942.
10. Levin, L., and Tyndale, H. H.: Concentration and Purification of the Gonadotropic Substance in Urine of Ovariectomized and Post-Menopausal Women, Proc. Soc. Exper. Biol. & Med. 34: 516, 1936.
11. Levin, L., and Tyndale, H. H.: The Quantitative Assay of "Follicle Stimulating" Substances, Endocrinology 21: 619, 1937.
12. Thomsen, O., and Pedersen-Bjergaard, K.: Nachweis Kleiner Mengen gonadotropien Hormons in Hard besonders von Normalen, Ztschr. f. Geburtsh. u. Gynäk. 112: 202, 1936.
13. Zondek, B.: Uber die Hormone des Hypophysenvorderlappens; Darstellung des Follikelreifungshormons (Prolan A)—Methodik der klinischen Harnalyse zum Nachweis des Prolan, Klin. Wchnschr. 9: 1207, 1930.

DOUBLE DISTILLATION IN ONE AND THE SAME FLASK*

F. RAPPAPORT, PH.D., PETAH TIQVA, PALESTINE

THE principle of the double distillation in one flask was described by us in 1934, and was put into practice by the construction of an apparatus for the preparation of double distilled water.† The described apparatus had the disadvantage of working only half automatically, as the water supply during the process of the distillation had to be regulated continually. These circumstances have induced us to construct the apparatus, reproduced in Fig. 1,

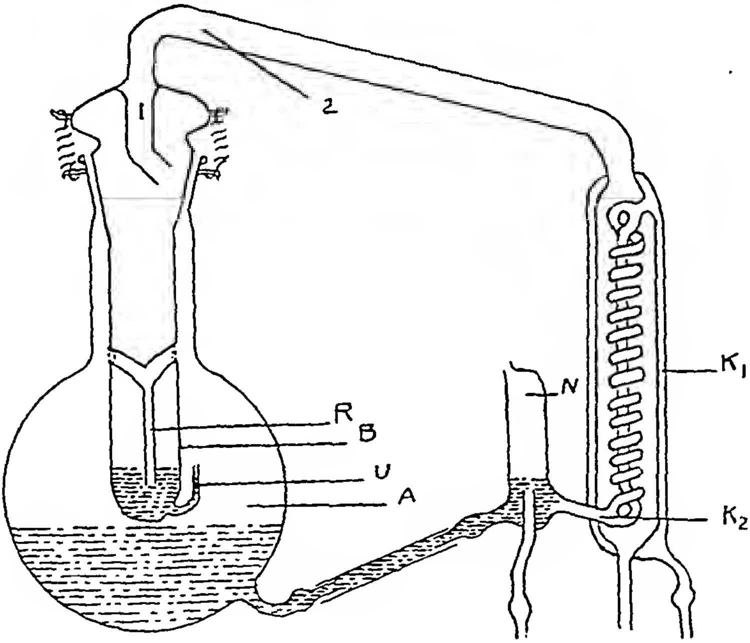


Fig. 1.

which guarantees a fully automatic double distillation. The apparatus consists of two distilling vessels (A and B), fitting into each other; A is heated by gas or electricity, B by steam created by A. The outer flask (A) is connected to a constant level arrangement (N), which allows a continuous water supply, thus replacing the water, evaporated in A. When the outer flask is heated, the steam gets into the inner distilling vessel (B) through Tube R, and from there via 1 and 2 into condenser K. By condensation in the condenser, part of the water runs down as singly distilled water. Another part, after condensation in Glass tube 1, runs back into vessel B and is condensed there, whereby the lower part of B up to the height of the drainage tube (U) is constantly filled

*From the Biochemical Laboratory of the Beilinson Hospital, Petah Tiqva, Palestine.
Received for publication, April 8, 1943.
†Rappaport, F.: Mikrochem. 15: 302, 1934.

with water. The constant heating by the steam, coming in from R, and the heat in flask A effect a renewed distillation. The drainage tube (U) acts in a way as a valve, as the condensed water which does not reach a second distillation flows off, thereby obtaining a constant pressure and regular boiling. The inner vessel (B) is connected by way of ground joint and elastic wire spirals with A, and *is directly melted to the double condenser,* K_1 *and* K_2.

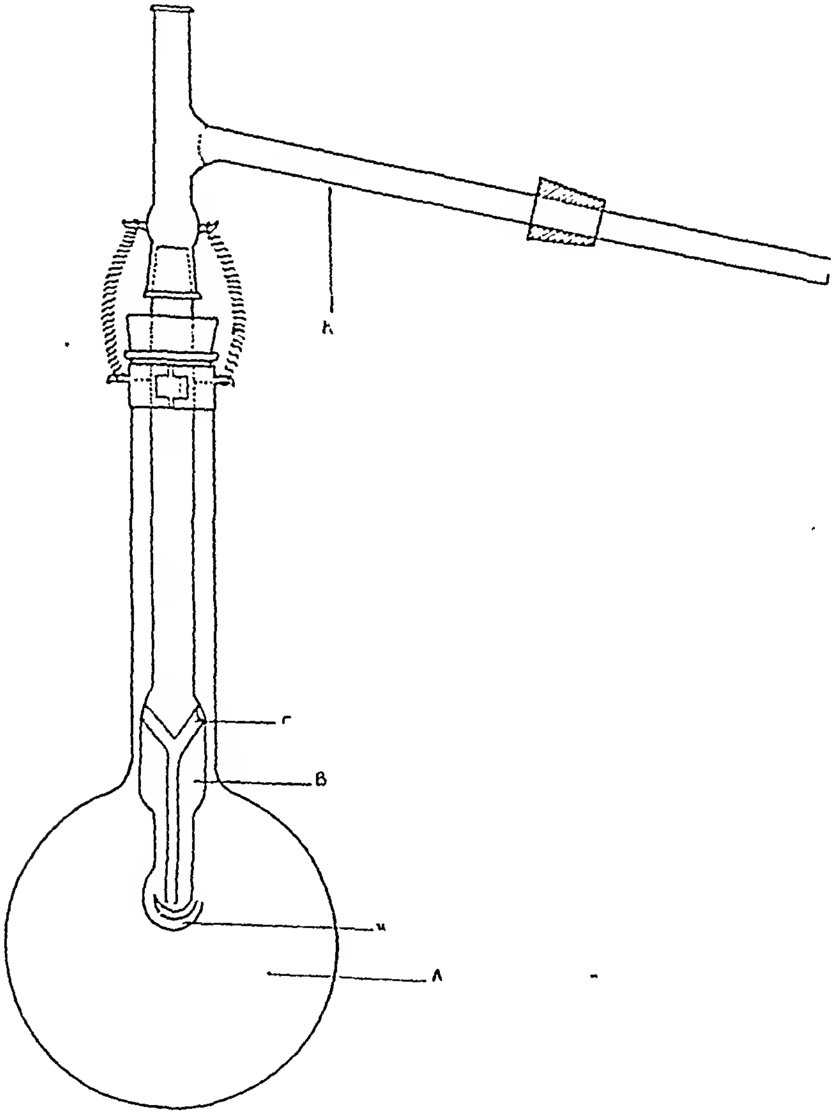


Fig. 2.

Thereby the apparatus becomes very handy and can be fixed on a stand. This apparatus supplies, provided appropriate dimensions are taken, approximately 1500 c.c. per hour. The water gained this way has a conductivity of $K = 1.5$ .10—6.

The principle of the one-sided double distillation can also be used for the rectification of different organic solvents with the aid of the apparatus,

reproduced in Fig. 2. The solvent is distilled from the outer round flask A into the inner vessel (B). Through continual heating by the steam coming through the Y-shaped tube (r), and also through steam present in bulb A, a second distillation into the inner vessel is effected. The water vapor gets into the distilling column (K) which is supplied with a thermometer and connected to a condenser. The column is connected by ground joint to vessel B which, according to requirements, can be fitted into flasks of different sizes by means of suitable stoppers. At the lower end of tube B a drainage tube (*u*) enables the condensates to run off during the distillation, thus effecting constant pressure in both vessels. The same principle of inserting two bulbs into one another

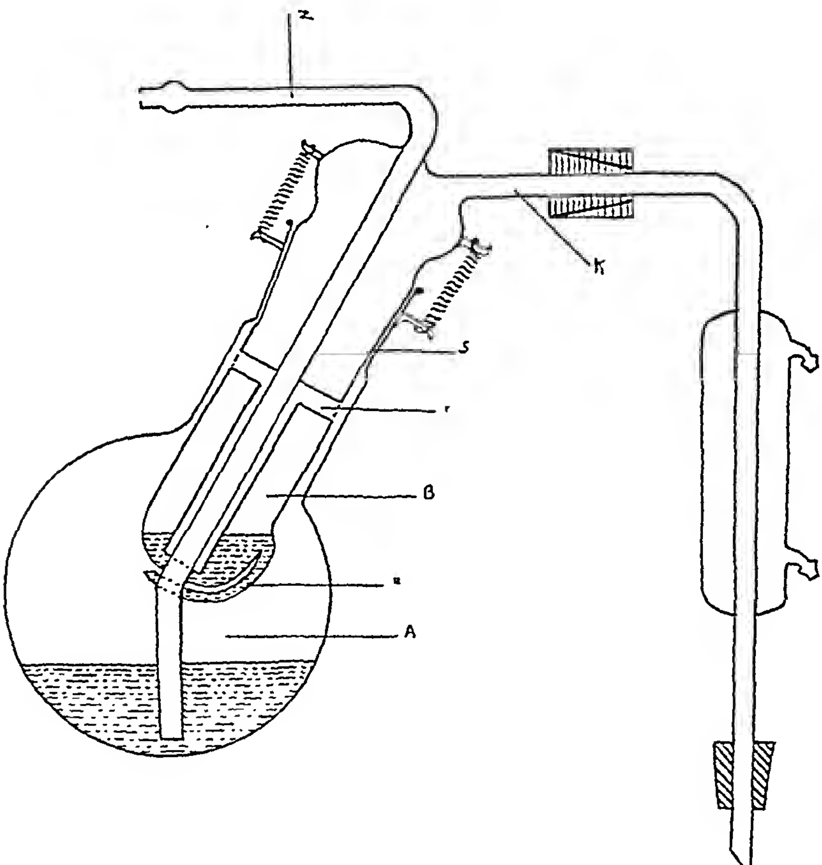


Fig. 3.

can also be profitably applied to steam distillation. (See Fig. 3.) Through tube z steam is led into the liquid that is immiscible with water in flask A. From there the steam reaches B over tube r. The condensing steam collects again on the bottom of the inner flask which communicates through drainage tube U with the outer flask and thereby stands under constant pressure. The oil-loaded steam, further produced in B, leaves through lead k into the condenser. This apparatus too may be furnished with stoppers instead of ground joints, and can thereby be connected with flasks of different sizes.

MICRODETERMINATION OF ACETONE AND DIACETIC ACID IN BLOOD*

F. RAPPAPORT, PH.D., AND B. BANER, PETAH TIQVA, PALESTINE

THE following method, which is based on the method of M. Ljungdahl,† allows the determination of acetone bodies accurately and quickly in 0.2 c.c. blood or serum by means of a simple and easily handled apparatus. The distillation apparatus (see Fig. 1) consists of three parts:

(1) The distilling flask (A), which is a 50 c.c. Erlenmeyer flask connected to the distilling headpiece by a ground joint.

(2) The distilling headpiece. When in use the connection is secured by a pair of elastic springs (*f*). The headpiece consists of a glass tube, the ends of which are bent downwards at an obtuse angle. The shorter side (a) is widened at its end to a semisphere (h) and is ground on to the distilling flask. The second and longer side (b) serves as a dropping tube and is surrounded by an external condenser tube for condensing the distillate. If there is no running water available, it is sufficient to fill the condenser with cold water once for each determination. The washing of the apparatus is done through the side tube (C), which is attached to the middle part of the distilling top piece. This tube (C) is extended to a capillary (D), running parallel to the axis. During the distillation, the tube is closed by a cork stopper. The glass rod, which is sealed on to the top of the semispheric ground joint and which ends closely above the water seal of the distilling flask, prevents boiling over.

3. The receiving vessel (B), a 50 c.c. Erlenmeyer flask, which is connected to the apparatus by a double clamp (S). One clamp holds the vessel; the second one is fixed to the condenser and can be moved so that the end of the distilling apparatus, according to requirement, is immersed into the received liquid or ends freely above it. To facilitate work during the distillation a hinge is fixed on the clamp carrier, thereby putting the flask in an oblique position and making it possible to shake it backwards and forwards as well.

REAGENTS

1. Diluted phosphoric acid solution. Two to three drops concentrated H_3PO_4 to 100 c.c. distilled water.

2. Iodine solution. 0.75 c.c. 1:10 N-Iodine solution and 5 c.c. 20 per cent sodium hydroxide are filled up to 50 c.c. with distilled water. (Prepared daily.)

3. Potassium iodide in crystals.

4. Concentrated hydrochloric acid diluted in equal parts with water in a dropping bottle.

5. 1:1000 n-sodium thiosulfate solution.

6. 0.25 per cent starch solution.

---

*From The Biochemical Laboratory of the Bellinson Hospital, Petah Tiqva, Palestine.
Received for publication, April 8, 1943.

†Eine Mikromethode zur Bestimmung des Total-Acetons im Blute Biochem. Ztschr. 96: 345, 1919.

PROCEDURE

Before beginning the determination, the distilling apparatus is rinsed with distilled water through the side tube (C) and the opening is closed quickly with the stopper, so that a small column of liquid in the capillary (D) closes the latter against the inside of the distilling headpiece. The ground neck part of the flask (A) is moistened with a bit of water. By means of the elastic springs the flask is fixed on the semisphere of the distilling headpiece. On the other side of the apparatus a flask (B) is clamped. Then water is heated in A and distilled into B until cleanness of the apparatus is fully assured. Now the analysis is started with the determination of the "blank value."

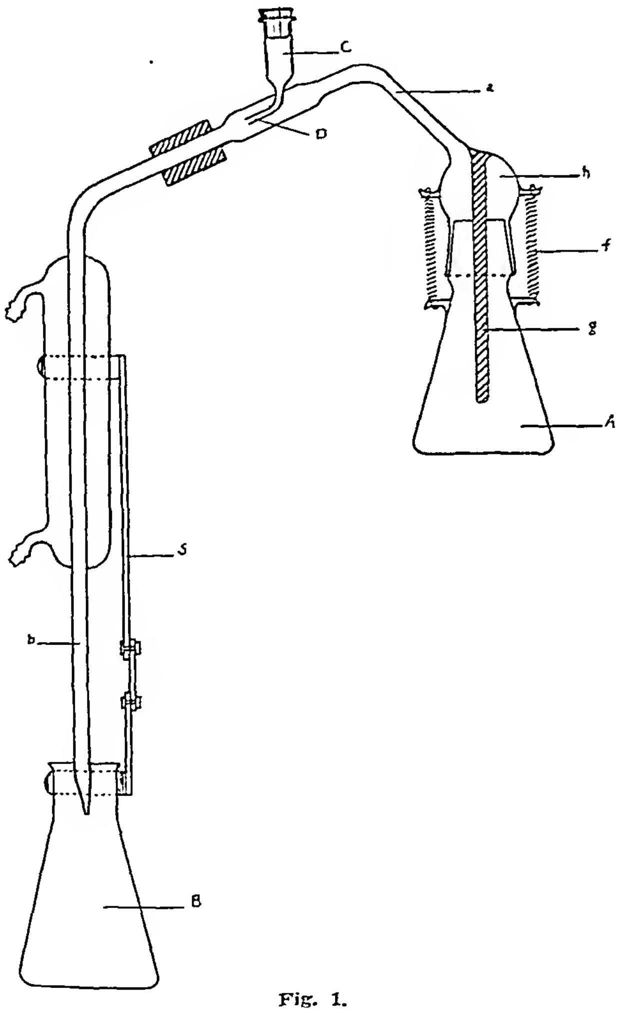


Fig. 1.

As receiving flask, flask B containing exactly 2 c.c. of the iodine solution (2) is fixed to the apparatus so that the distilling tube is immersed in the fluid. On the other side a distilling flask (A) containing 4 c.c. phosphoric acid is fixed in the manner explained above, and is heated by a small flame. The phosphorie acid is to be measured in a small, narrow-measuring cylinder in order to avoid mistakes arising from traces of alcohol, ether, or acetone left in the pipettes. When the contents of the flask have boiled for three minutes, flask

B is pushed down, so that the distilling tube is no longer immersed and goes on boiling for only another minute. Eventually distilled water is rinsed through the opening (C).

Some crystals of potassium iodide, hydrochloric acid, and a few drops of starch solution are put into flask B. It is shaken and then titrated with 1:1000 n-sodium thiosulfate solution. The value thus found is the "blank value." It must be equal to the value found when adding potassium iodide, hydrochloric acid, and starch solution to 2 c.c. of iodine solution in the same way in a receiving flask and when titration is carried out without any distillation. When this is not the case, the distillation must be repeated. Once the "blank value" is found, the actual determinations can be carried out, one after the other. This is done by adding, using the precautions necessary in exact analytic determinations, 0.2 c.c. of the blood or serum to be examined to the measured amount of phosphoric acid in flask A, which are attached to the apparatus in succession. Distillation is carried out just the same as described for the determination of the "blank value." Titration of the iodine solution in flask B, carried out subsequently, gives the full values.

CALCULATION

"Blank value" minus sample value multipled by 4.83 gives the acetone content in mg. per 100 c.c.

Example:

Blank Value	2.72
Sample Value	1.34
Difference	1.38

$1.38 \times 4.83 = 6.6654$ mg. per 100 c.c. acetone.

The figures given in the accompanying tables prove the utility of the method.

1. Amount of c.c. n/1000 thiosulfate solution required with diluted acetone solution.

Without Distillation	With Distillation
0.45	0.44
0.90	0.91
1.34	1.33
1.79	1.77

2. Blood and serum to which measured amounts of acetone were added always gave values that corresponded to the amount of acetone found in the blood plus that added to it.

Example:

Blood	0.78 c.c. n/1000 of thiosulfate solution
Acetone Solution	1.34 c.c. n/1000 of thiosulfate solution
Calculated Amount	2.12 c.c. n/1000 of thiosulfate solution
Blood Plus Acetone Solution (Value Found)	2.10 c.c. n/1000 of thiosulfate solution

SUMMARY

A method is described which allows the determination of the content of acetone in 0.2 c.c. blood or serum with great accuracy and a simple apparatus.

This work was begun in Vienna and completed in Palestine.

BOOK NOTICES

Chemotherapy of Gonococcic Infections*

TREATMENT of gonococcic infections with the sulfonamide drugs is presented from the standpoint of a streamlined program of practical value. Choice of drugs, dosage, and toxicity are fully discussed. Special mention is made of the infectious carrier and the sulfonamide resistant case. Various methods of treatment such as fever therapy combined with a sulfonamide and vaccine combined with a sulfonamide are discussed, as are treatments of specific complications.

Of particular value to the busy practitioner is the omission of discussions of history, bacteriology, pathology, and endoscopic examination and treatment.

The problem of diagnosis and determination of cure in both male and female are stressed. At the end, certain investigative problems are briefly presented, and case outlines are given to illustrate points in diagnosis, treatment, and complications.

The book has a high practical value to the practitioner as well as to the specialist, in its adequate, concise presentation.

Blood Groups and Transfusion†

THE third edition of Dr. Wiener's book *Blood Groups and Transfusion* has much new material, necessitated by the many advances in whole blood and plasma transfusion, as developed in the last few years. The presentation is very comprehensive. It includes discussion of blood groups and technique of grouping, selection of donors, indications for transfusions, methods of transfusion of whole blood, plasma, and serum, reactions and complications, and the organization and use of blood banks. There is a chapter on the history of transfusion, and sections are devoted to the heredity of blood groups, the newly recognized Rh factor, group-specific substances in organs and body fluids other than the blood, medicolegal application of blood tests, and technique for the identification of blood stains. There is a very interesting chapter on anthropologic studies of blood groups and on evolution of the human blood groups.

This volume should have a wide field of usefulness.

The Physiological Basis of Medical Practice‡

SINCE its first printing in January, 1937, Best and Taylor's *Physiological Basis of Medical Practice* has had three American editions, one Spanish, one Portuguese, and has had a total of twelve printings. Numerous recent advances in the application of the physiologic principles to clinical medicine, many of which have been occasioned by the exigencies of war, have necessitated many additions throughout the book. As formerly, the volume is primarily a work on applied physiology, useful not only as a text for medical students but as a reference volume for clinicians.

*Chemotherapy of Gonococcic Infections. By Russell D. Herrold, B.S., M.D., Associate Professor of Surgery (Urology), College of Medicine, University of Illinois, Chicago, Illinois. Cloth, 137 pages, $3.00. The C. V. Mosby Company, St. Louis, 1943.

†Blood Groups and Transfusion. By Alexander S. Wiener, A.B., M.D., Serologist and Bacteriologist in the Office of the Chief Medical Examiner of New York City; Head of Transfusion Division, The Jewish Hospital of Brooklyn, New York. Cloth, 439 pages, $7.50. Charles C Thomas, Springfield, Ill., Baltimore, Md., 1943.

‡The Physiological Basis of Medical Practice. A University of Toronto Text in Applied Physiology. By Charles Herbert Best, M.A., M.D., D.Sc. (Lond.), F.R.S., F.R.C.P. (Canada); Professor and Head of Department of Physiology; Associate Director of the Connaught Laboratories; Research Associate in the Banting-Best Department of Medical Research, University of Toronto, and Norman Burke Taylor, M.D., F.R.S. (Canada), F.R.C.S. (Edin.), F.R.C.P. (Canada), M.R.C.S. (Eng.), L.R.C.P. (Lond.), Professor of Physiology, University of Toronto. Third Edition. Cloth, 1942 pages, $10.00. A William Wood Book. The Williams & Wilkins Company, Baltimore, 1943.

Allergy, Anaphylaxis and Immunotherapy*

THE contents of Ratner's book, *Allergy, Anaphylaxis and Immunotherapy* is better described in the subtitle, "A Treatise Presenting the Fundamental Principles and Practice Governing the Use of Antisera, Vaccines, Toxoids, Blood Transfusions, Blood Substitutes and Sulfonamides, in the Prevention and Treatment of Infectious Diseases and of the Allergic Phenomena Resulting From Their Use."

It may be described as a book on immunotherapy with homologous and heterologous sera, vaccines, and sulfonamides, with the discussion of allergic and other responses. The analysis of experimental work in the field under discussion, including the author's own contributions, is splendidly arranged and completely up-to-date.

The book will be of great value to allergists, immunologists, serologists, and is especially opportune because of its usefulness to all physicians who have occasion to give whole blood or plasma transfusions.

There is a section dealing with specific immunotherapy in the various infectious diseases.

CORRESPONDENCE

In view of the need for pathologic material in under-graduate and graduate education, the Committee on Pathology of the National Research Council urges that all who have suitable anatomic specimens forward them to the Curator of the Army Medical Museum, Washington, D. C., for correlation and distribution to other central agencies and to teaching institutions. Material from the following is particularly wanted: the malarial diseases, bacillary dysentery, endamebiasis, the schistosomiases, filariasis, the trypanosomiases, the relapsing fevers, the leishmaniases, the rickettsial diseases, yellow fever, cholera, plague, and yaws. On application to the Curator, arrangements for transportation will be made.

*Allergy, Anaphylaxis and Immunotherapy. Basic Principles and Practice. A Treatise Presenting the Fundamental Principles and Practice Governing the Use of Antisera, Vaccines, Toxoids, Blood Transfusions, Blood Substitutes and Sulfonamides, in the Prevention and Treatment of Infectious Diseases and of the Allergic Phenomena Resulting From Their Use. By Bret Ratner, M.D., Clinical Professor of Pediatrics, New York University [illegible] Visiting Pediatrician and Director of Pediatrics, Sea View Hospital; Assoc[illegible] dren's Medical Service, Bellevue Hospital; Consultant Pediatrician, French [illegible] cloth, $8.50. The Williams & Wilkins Company, Baltimore, 1943.

The Journal of Laboratory and Clinical Medicine

Vol. 28 December, 1943 No. 15

CLINICAL AND EXPERIMENTAL

THE PRODUCTION OF STAPHYLOCOCCUS ANTITOXIN*

James B. Weaver, M.D., Mary Whelan Tyler, M.D., and Doris K. Schuerman, A.B., Kansas City, Kan.

THE tissue, or tissues, responsible for the production of staphylococcus antitoxin are not known, but it is generally assumed that such production takes place somewhere in the hemopoietic system. In an effort to throw some light on this problem, the authors have done two types of experiments. First, various components of the hemopoietic system were "blocked out" in experimental animals and the immune reaction of these animals to staphylococcus toxoid as antigen was observed. Second, extracts from various tissues of immunized animals were examined for the presence of antitoxin as indicated by the titer of antihemolysin.

METHODS

White rabbits of five to six pounds each were used in all experiments. Stimulation of antibody production was produced by subcutaneous injection of Lederle's staphylococcus toxoid three times a week, the initial injection being 0.1 c.c., and the dosage increased by 0.1 c.c. at each injection until a total of 1 c.c. was given at one time. The injection period, therefore, covered a little more than three weeks. Titers of antihemolysin were determined twice a week. This procedure was followed in each series of experiments, and in some series total leucocyte and differential counts and hemoglobin determinations were done daily or three times a week as indicated. In most cases the animals were sacrificed at the end of the study period, and sections of bone marrow from typical long and flat bones, spleen, and liver were taken for microscopic study. The results of the study of the microscopic slides will be reported in another paper.

*From the Hixon Research Laboratory of the University of Kansas Medical School.
This work was financed by the Kansas State Board of Health, through funds derived from the U. S. Public Health Service.
Received for publication, May 17, 1943.

CONTROLS

A series of six control animals was run, and, in addition, two controls were studied concurrently with each series of experiments, making a total of nineteen. The results obtained in each individual series were compared against this relatively large number of control animals.

Seventeen of the 19 control rabbits had no initial antibody titer, while two had 0.6 international units of antihemolysin per c.c. before toxoid injections were begun. These rabbits varied somewhat in the length of time required for the response to stimulation, the antibodies appearing in the serum from the eleventh to the twenty-ninth day after the first injection, the average for the whole series being the eighteenth day. The highest concentration of antibodies in the serum was reached at intervals between the eighteenth and the fortieth day, the average being twenty-two.

BENZENE

It has long been known that benzene has a depressant action on the bone marrow, affecting primarily the elements having to do with the formation of the leucocytes. Kracke[1] has shown that benzene given subcutaneously has a marked depressant action on the white blood cells, particularly the granulocytes, and that the erythrocytes are little affected. This is true, except that there is a definite drop in hemoglobin after benzene injections have been continued for some time.

The authors have observed that rabbits have a variable individual resistance to this substance, so that many of them died quickly with a very low leucocyte count, or in an apparently very toxic condition when the white cell count was not at what could be considered a critical level. Autopsy on these animals showed nothing grossly abnormal. The final series consisted of 9 rabbits which survived long enough to receive the full dosage of toxoid. In addition, there were 4 rabbits which received from 0.5 to 0.7 c.c. of toxoid at one time. The general procedure in this series was to inject 1 c.c. of benzene per kilo of body weight subcutaneously daily until the leucocyte count reached 3,000 or less, and then to inject at intervals the amount of benzene necessary to keep the leucocytes at approximately 2,000. When the leucocytes had been sufficiently depressed, toxoid injections were begun. It was not uncommon for the level of the total white cells to reach relatively high levels, probably due to an effort on the part of the bone marrow to overcome the rapid destruction brought about by the benzene.

Of the 9 rabbits in this series, 7 had no initial titer and did not begin to show serum antibodies until twenty to forty-one days after toxoid injections were begun (average, twenty-seven days), and reaching the height in from twenty to forty-eight days (average, twenty-six). Three never did develop a titer. Chart 1 shows four graphs which are typical of the results obtained in this series. Observe the extent of the depression of the leucocytes produced by benzene injections, particularly in the case of Rabbit 12, and the delay in the appearance of antibodies, as well as the small total amount of antihemolysin present. As shown in Chart 2, in which the average curves of serum antihemolysin for both controls and benzene series are plotted, there is a distinct delay in the appearance of antibodies in the benzene series, and the total content

is much lower than that of the control series. Rabbit 12 shows the combined effect of benzene and toxoid very well. This rabbit was studied for somewhat over three months after benzene injections were begun. The leucocyte count was kept at a very low level most of the time, with the occasional elevations mentioned previously. This rabbit had a negative titer for forty-one days after toxoid injections were begun, in spite of the relatively heavy dose of antigen given. In this case the total dose was carried to 1.5 c.c. and an additional 1 c.c. was given at weekly intervals for two injections. It was not until a week following the last injection that a positive titer was demonstrated. The rabbit died with a leucocyte count of 200.

At autopsy these animals showed nothing grossly abnormal except for a very pale bone marrow. Fig. 1 shows bone marrow from normal control rabbits. In contrast, Fig. 2 shows the hypoplastic bone marrow produced by repeated injections of benzene.

CARBON BLOCK

In an effort to block the reticulo-endothelial system, an 8 per cent suspension of Higgins Waterproof India Ink was injected intravenously and intraperitoneally daily in amounts of 20 c.c. by each route. Injections were continued throughout the study. A suitable interval, based partly on the elevation of the total white count but principally on the appearance in the peripheral circulation of considerable numbers of nucleated red cells, was allowed to elapse before toxoid injections were begun. From 200 to 350 c.c. of the suspension were injected, as a rule, before giving toxoid. Cannon et al.[2] have pointed out the importance of continuing the daily injections, particularly intravenously, throughout the period of study and giving the blocking material in sufficiently large amounts to overcome the tendency to stimulation produced by smaller amounts. As much as 1,220 c.c. of the 8 per cent suspension of ink has been given a rabbit during the period of study. Cannon[2] has shown that the amount of fluid is not significant, for he produced no effect in the rabbits to which he gave isotonic saline by the same routes and in the same quantity as his blocking material.

Of the 9 rabbits used in this series, 7 had no initial antihemolysin titer, and the average time of the appearance of antibodies after toxoid injections was twenty-three days (ten to forty). In these, the maximum titer was obtained on an average of twenty-seven days (fourteen to fifty). Chart 3 shows curves for total white cells and antibody content obtained in four typical animals of this series. Note the marked elevation of the total leucocyte count, the delay in appearance, and the low content of antibodies in the serum. Differential counts showed nothing of particular significance except the appearance of large numbers of nucleated red cells and phagocytosis of carbon particles by the polymorphonuclear leucocytes and monocytes. Hemoglobin showed a steady and marked drop as the block was continued. At the end of the experimental period, the hemoglobin content was well below 7 Gm. per cent in all cases.

Chart 2 shows composite curves plotted against the controls. It may be observed that the curve for the blocked animals is much flatter than that for the controls, that the antibodies appeared later and did not rise to so great a quantity. Of the two rabbits which showed an initial titer, one (39) showed a

Fig. 1.

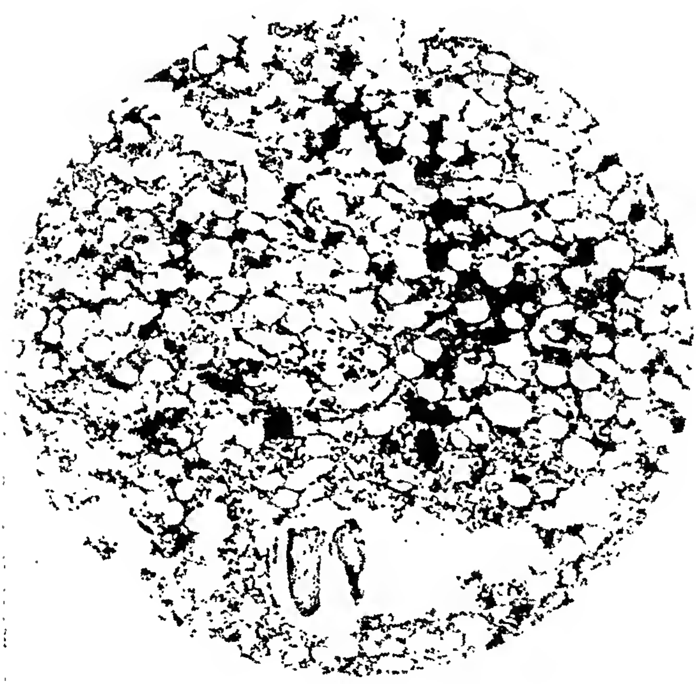


Fig. 2.

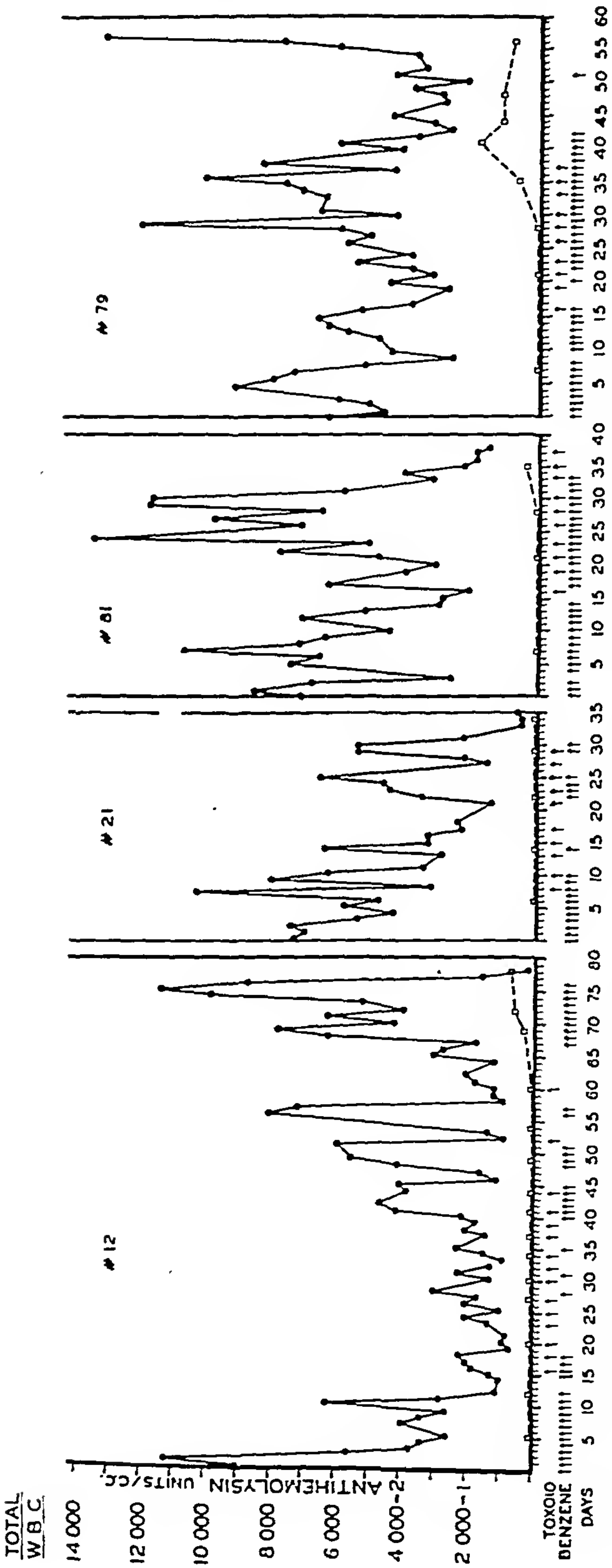


Chart 1.—Total leucocyte count and serum antihemolysin in four typical rabbits in which the bone marrow has been depressed with benzene.

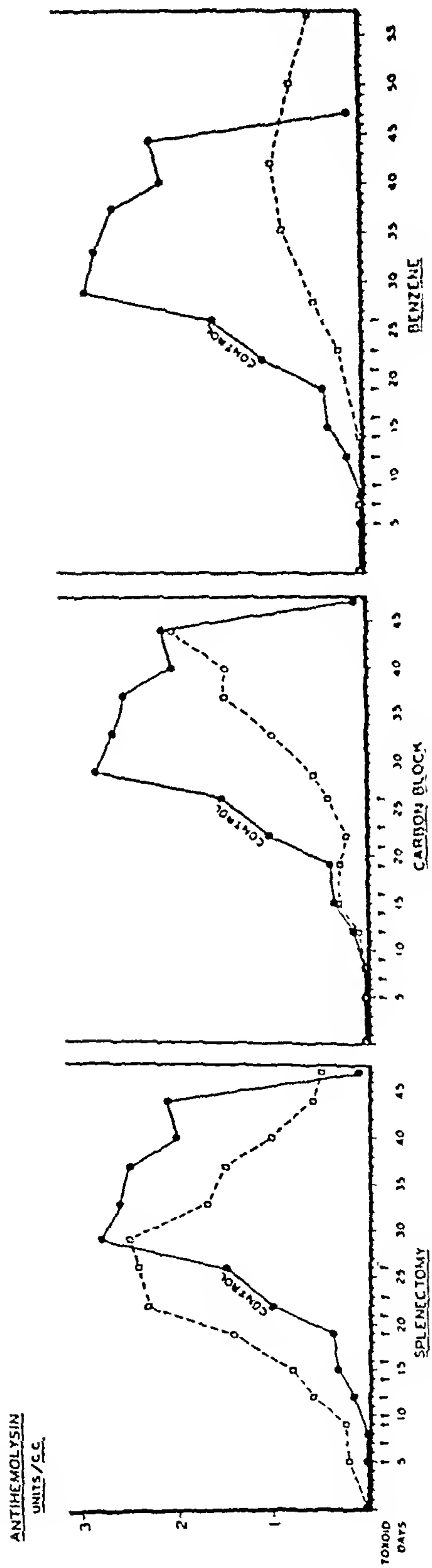


Chart 2.—Average curves of serum antihemolysin in animals blocked by carbon and benzene and in splenectomized and control animals.

diminution in serum antibodies after the ink injections were begun, while the other (42) showed a steady rise.

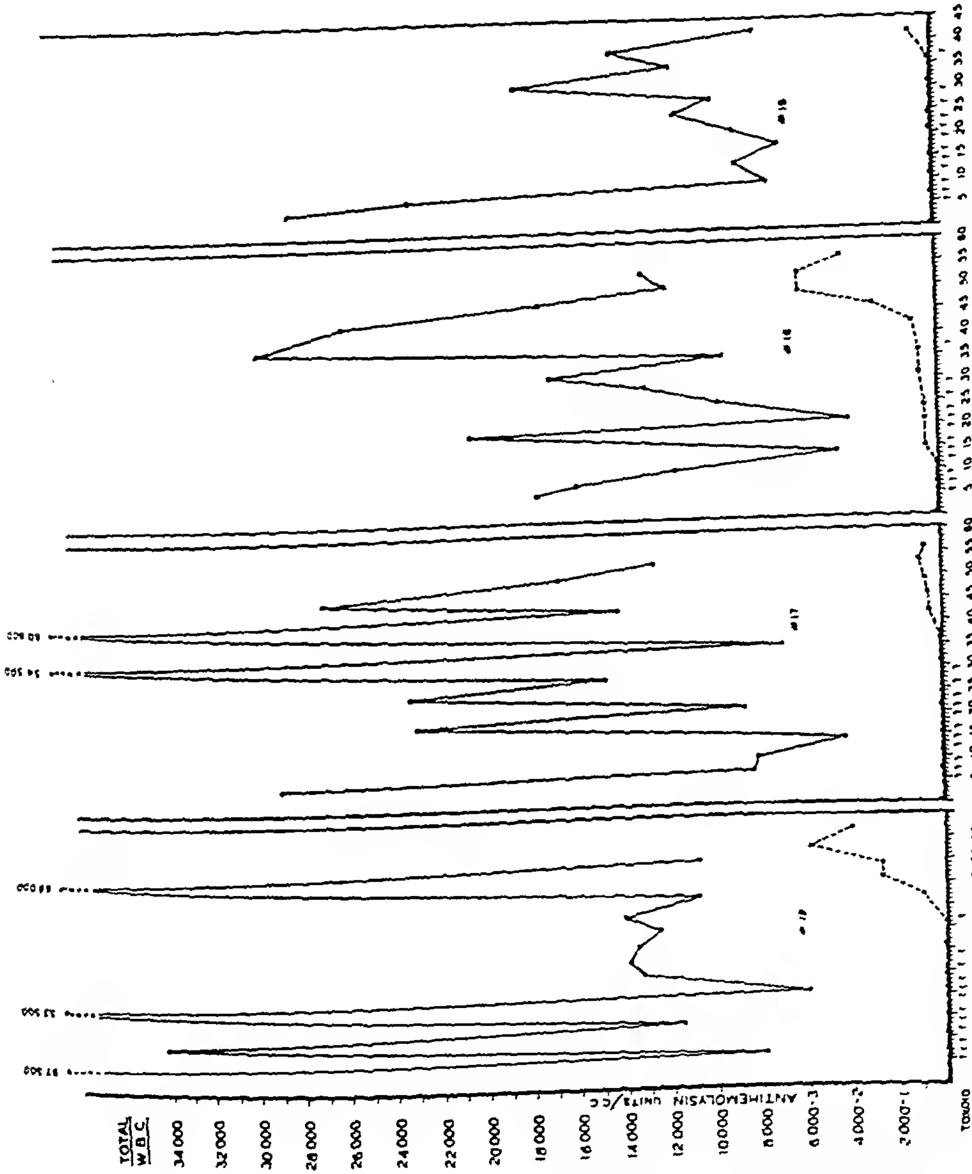


Chart 3.—Total leucocyte count and serum antihemolysin in four typical rabbits in which the hemopoietic system had been blocked with carbon.

The total leucocyte count in the blocked animals was usually relatively high, being well above that of the control animals and increasing to as much as 96,000 in some instances (Chart 3). The animals were killed after a definite decline of serum antibody content occurred, and at autopsy all lymphoid tissue was found to be stained black, the liver and spleen were greatly hypertrophied, the spleen having increased in weight approximately ten times that of the normal animals. The bone marrow was intensely black, the omentum thickened and enlarged. Grossly, the kidneys showed no evidence of having taken up carbon.

SPLENECTOMY

Splenectomy was performed on 11 animals. Three weeks were allowed to elapse following operation before toxoid injections were begun, in order that the

animals might fully recover from the effects of the surgical procedure. Chart 4, where four typical curves are shown, and Chart 2 show the results obtained. It may be noted that the curve for antibody production is similar to that of

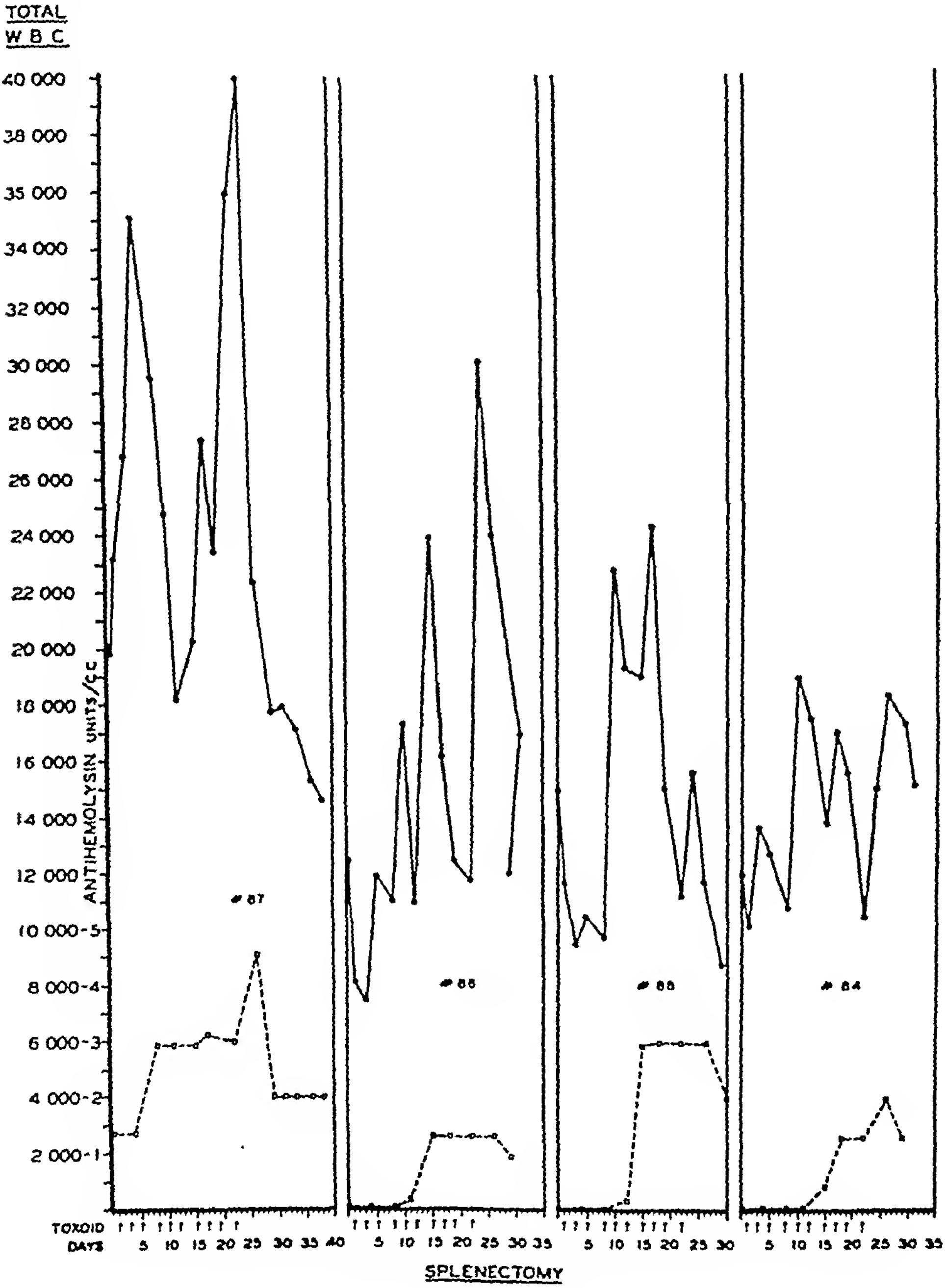


Chart 4.—Total leucocyte count and serum antihemolysin in four typical rabbits from which the spleens had been removed.

the controls in height and shape, differing only in the time of the first appearance of antibodies in the serum. In case of the splenectomy series, antibodies appeared sooner. Analysis of the individual results shows that antibodies appeared

in the serum in two to sixteen days, with an average of eleven days, and the height was reached from the tenth to the twenty-third day (average, nineteen days).

In order to rule out the effect of surgical procedure, nephrectomy was performed on four rabbits. Toxoid injections were begun after a three-week period. The production of antihemolysin showed no particular difference from that in control animals.

Chart 5 is a composite of all curves and shows very clearly the delaying effect of benzene and carbon block on the appearance of antibodies in the serum.

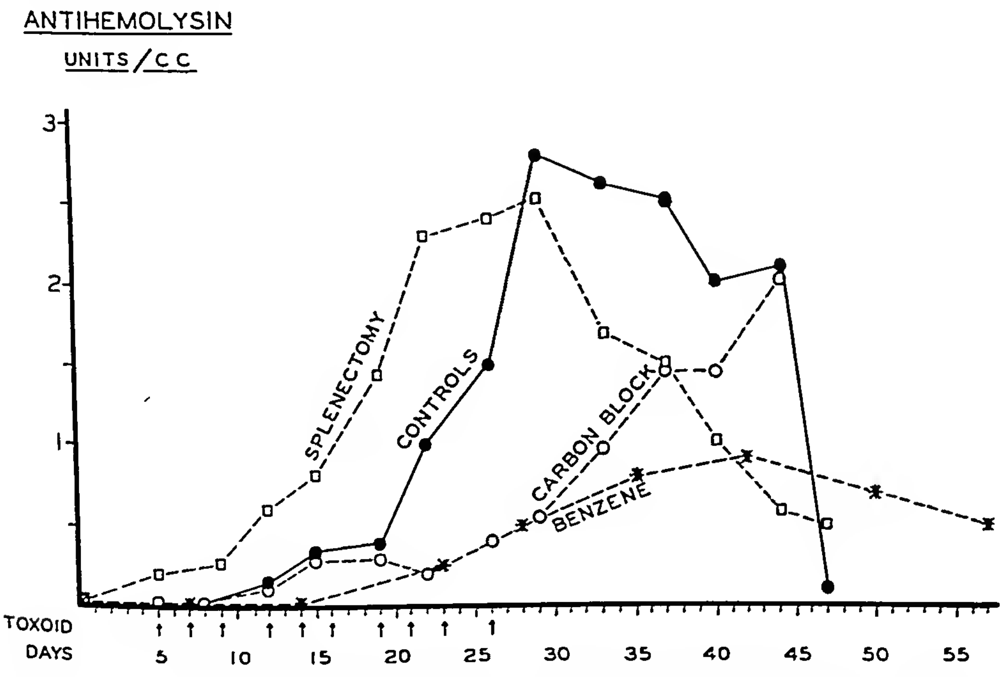


Chart 5.—A composite of all curves showing the depressing effect of carbon and benzene block and the apparent transitory stimulation due to splenectomy.

TISSUE EXTRACTS

It was thought that it might be interesting to study the organs rich in lymphoid tissue for their antibody content. Cary[3] and Motahashi[4] determined tissue antibodies after injecting foreign erythrocytes, by means of extraction with 50 per cent glycerin (14 to 1 dilution) in the incubator for five to six days. This procedure was followed, but we were unable to demonstrate antibodies in the spleen, bone marrow, or liver of sacrificed animals with appreciable known quantities of antitoxin in their blood serum. Undoubtedly this was due to the relatively large dilution recommended. The following procedure was therefore devised.

The animals were exsanguinated and the tissues removed immediately after death, placed in the freezing unit of the refrigerator for twenty-four hours, then pressed through a fine sieve. Two grams of tissue were extracted with 2 c.c. of 0.85 per cent saline for five days in the refrigerator. The tubes were then

centrifuged at high speed, and the supernatant fluid was used for the antihemolysin determinations. The procedure from this point was the same as that used for serum antibody determinations.

By this means we have demonstrated the presence of antihemolysin in bone marrow in 19 of the 56 rabbits in which antibodies were present in varying

TABLE I

ANTIHEMOLYSIN—U/GM. OF TISSUE

RABBIT	SERUM	BONE MARROW	LIVER	SPLEEN	MUSCLE	R.B.C.
12	0.34	0	0	0	--	--
16	2.98	0	0	0	--	--
17	0.34	0	0	0	--	--
18	0.44	0	0	0	--	--
19	2.98	0	0	0	--	--
21	0	0	0	0	--	--
23	2.98	0	0	0	--	--
24	6.8	0	0	0	--	--
25	0	0	0	0	--	--
31	0	0	0	0	--	--
32	0.34	0	0	0	--	--
36	0	0	0	0	--	--
41	0	0	0	0	--	--
50	0.34	0	0	0	--	--
51	16.8	2.0	0	0	--	0
53	0.26	0	0	0	--	--
62	0.38	0.18	0	--	--	--
64	0.38	0	0	--	--	--
65	0.18	0	0	--	--	--
66	2.0	0.38	0	--	--	--
67	1.34	0	0	0	--	--
68	1.34	0.18	0	0	--	--
69	0.44	0.18	0	0	--	--
79	0.38	0	0	0	0	--
82	0.58	0	0	0	0	--
83	0.38	0	0	0	0	--
84	1.32	0.38	0	--	0	--
87	2.0	0.58	0	--	0	--
88	2.0	1.32	0	0	0	--
115	1.34	0	0	0	0	--
116	2.0	0	0	0	0	--
117	1.34	0	0	0	0	--
132	7.0	0.89	0	0	--	--
152	0.88	0	0	0	0	0
153	0.26	0	0	0	--	0
155	1.32	0	0	0	--	0
157	0.88	0	0	0	--	0
158	1.32	0.34	0	0	--	0
159	1.32	0	0	0	--	0
160	2.98	0	0	0	--	0
161	2.0	0	0	0	--	0
164	2.98	0	0	0	--	0
166	1.32	0	0	0	--	0
167	3.98	1.2	0	0	--	--
168	2.0	1.2	0	0	--	0
169	4.48	2.66	0	0	--	--
170	3.98	1.2	0	0	--	--
171	2.0	0.8	0	0	--	--
172	2.0	0	0	0	--	--
173	3.98	0	0	0.89	--	--
174	4.48	1.2	0	0	--	--
175	1.33	0	0	0	--	0
176	3.98	0.4	0	0	--	0
177	0.6	1.2	0	0.6	--	--
178	0.6	0	0	0	--	--
179	2.0	1.2	0	0	--	0

quantities in the serum. Table I gives the results. Liver, spleen, and bone marrow were studied, and in a few cases, muscle tissue determinations were made as controls. As can be seen in Table I, antibodies were demonstrated only in bone marrow, with the exception of two instances in which small amounts were found in the spleen.

COMMENT

Several factors must be considered in an evaluation of the preceding results. A marked variation in immune response was noted in many of the experimental animals. The cause of this phenomenon is not known, but is usually ascribed to individual characteristics (whatever they may be). The carbon block procedure is not considered to be too efficient. The effect of the carbon was a marked stimulation of leucocyte production instead of depression, and the absorption of carbon by component cells to neutralize their activity, was relied upon to effect the block. It can be readily seen that in this method it is almost impossible to keep the stimulation-block mechanism in complete balance, and it is very easy for the pendulum to swing to the stimulation side.

Benzene is considered a more efficient agent than carbon, but its action is more selective as it apparently affected only the bone marrow. However, its efficiency was somewhat impaired as a result of the toxicity of the drug. When too much benzene was given, the animal died, but if too little was given, bone marrow stimulation ensued.

Splenectomy is considered efficient. The operative procedure might have enhanced antibody production. However, this factor seems to have been ruled out by the results obtained after nephrectomy.

The tissue extract experiments are considered moderately accurate. Care was taken to thoroughly exsanguinate animals so that antibody concentration in the serum would not be measured. The results in Table I are evidence of the success of the method.

It may, therefore, be said that while no definite conclusions may be drawn, yet there is some evidence that staphylococcus antitoxin is produced in the bone marrow.

REFERENCES

1. Kracke, Roy R.: The Experimental Production of Agranulocytosis, Am. J. Clin. Path. 2: 11, 1932.
2. Cannon, Paul R., Baer, Raymond B., Sullivan, F. L., and Webster, J. R.: The Influence of Blockade of the Reticulo-Endothelial System on the Formation of Antibodies, J. Immunol. 17: 441, 1929.
3. Cary, W. E.: The Relation of Hemophages to Antibody Production, J. M. Research 43: 399, 1922.
4. Motahashi, Shinzo: Fixed Tissue Phagocytosis, J. M. Research 43: 419, 1922.

HUMAN PLASMA AND SERUM TOXICITY

David State, M.D.,* Minneapolis, Minn., and
Milton Levine, M.S., Ph.D.,† Chicago, Ill.

REACTIONS have been reported for both serum and plasma following intravenous administration in humans and animals. The reports are, however, at variance as to the frequency of their occurrence and their severity. Many observers prefer plasma, whereas others by continued use seem to prefer serum for human transfusions. The literature on the subject yields many conflicting observations on the relative merits of the two fluids.

Strumia and his co-workers[1] are firm believers in the value of plasma because of the reactions encountered with serum: "we continue for several reasons[2] to give preference to plasma." McGuiness, Stokes, and Mudd[3] prefer plasma because of the severe reactions observed following the use of lyophile immune serum. Ravdin[4] states, "The frequency with which reactions have occurred following the injection of lyophile serum makes us hesitate to suggest the general acceptance of this material." Meakins[5] is but one of many others reporting serum transfusion reactions.

On the other hand, Levinson and his co-workers[6] continue to use serum and have no doubts as to its safety. "No reactions were observed or need be anticipated if serum is properly prepared." Mellanby[7] urges the use of serum, as do also Self and Scudder.[8] For ease in preparation, the latter prefer serum despite numerous reactions following such transfusions.

It would seem, therefore, that serum is suspected of causing more reactions than plasma, but that some workers prefer serum for other considerations than that of reactions, and these workers either deny that reactions should occur[6] or are of the opinion that the reactions are not important.[8]

The references to plasma reactions are not as copious as for serum. Because of this, many workers have assumed that reactions with plasma do not occur. However, such reactions have been reported. Stephenson and co-workers[9] described a plasma reaction with chills and fever. Self and Scudder[8] observed 4.9 per cent reactions with type specific liquid plasma, 8.2 per cent with pooled liquid plasma, and 5.6 per cent with dried pooled plasma, in a small series of cases. Elliott[10] reported 482 injections of plasma with three reactions. Polayes[11] and Levine and State[12] have given detailed case studies of plasma reactions.

There are many reports of reactions without detailed description as to number or type. Mahoney and his collaborators[13] simply state that, "there have been very few reactions," which indicates that they did observe them. Strumia et al.[14] talk about a "mild urticarial reaction," but conclude that the use of plasma "is simple, safe, and free of reactions."[2]

*Department of Surgery, University of Minnesota.

†Hektoen Institute for Medical Research, Cook County Hospital, and the Department of Bacteriology and Public Health, University of Illinois, Chicago, Illinois.

Received for publication, May 10, 1943.

It is probably correct to conclude that reactions do occur with both serum and plasma. As to serum, early experimentation may explain part of its excessive toxicity. From the time Bowditch[15] first perfused the frog's heart with serum in 1871, much work has been done on serum and plasma transfusion and perfusion in animals. Moldovan[16] in 1910 reviewed the literature up to that period and concluded that serum was more toxic than plasma. He attributed this to a "fibrin ferment" formed in strictly fresh defibrinated blood or blood serum. This ferment was destroyed in about half an hour. For later reviews on the subject, the reader is referred to the papers by Janeway, Richardson, and Park,[17] and Amberson.[18]

During the course of several thousand plasma transfusions at the University of Minnesota Hospitals, we observed reactions frequently enough[19] to invite more detailed observation and experimentation. Since we had been using group specific plasma routinely in the hospital because of ease in preparation, it was possible to make our observations without the complicating factors involved in pooling. In addition, pooling was not practiced routinely, because the main reason for doing so (neutralization of antibodies) had in our opinion never been conclusively established as necessary.

REACTIONS DUE TO A AND B SUBSTANCES

Shortly after the decision was made to study these reactions experimentally, we[12] observed a reaction to plasma in a patient who had been given a routine transfusion on the surgical wards. This patient, G. H., was of blood group O. He developed chills, severe dyspnea, and cyanosis following the intravenous administration of 150 c.c. of undiluted group A plasma. The possibility of incompatible agglutinins in donor plasma was eliminated, since the red cells of the recipient were of group O and hence inagglutinable by the administered plasma. By all theoretical immunologic considerations this group A plasma should have been innocuous. We decided to skin test the patient with the plasma. Previously we[19] had suggested the possible value of such a procedure, knowing well the difficulties of skin tests in general and yet recognizing its invaluable aid in testing for sensitivity to tetanus, pneumococcus, diphtheria, and other antisera.

We found that not only did this patient give a positive skin test with the reacting plasma, but also with three other group A plasmas and one AB plasma, but not with B or O plasmas. The patient was subsequently given 100 c.c. of another group A plasma with resultant chills, cyanosis, and dyspnea. Group O and group B plasmas given intravenously gave no reactions.

From the above and similar cases, we assumed some relationship between the reactions and the blood group of the administered fluid.

Moss[20] in 1910 demonstrated that the hemolytic action of A serum on B cells can be prevented by mixing A serum with B serum. Schiff[21] later found that serum of group A blood contained soluble A substance which seemed to have the same antigenic structure as A substance in the red cells. Levinson and his co-workers[22] found that mixing of A and B sera caused neutralization of the isoagglutinins in both sera. Lubinski[23] attributed this phenomenon to a nonspecific factor, but Aubert and his associates[24] ascribed the neutralization to the dissolved A and B substances found in serum and plasma of the corre-

sponding blood groups and were able to confirm this neutralization by pooling and by the addition of purified A and B substances prepared by Witebsky and his co-workers.[25]

We felt that these dissolved A and B substances might be responsible for the phenomena described. Fortunately we were able to obtain the purified A and B substances from Witebsky, and we used this material to check our hypothesis.

We can summarize our findings as published or still in press. Patients sensitive to the A or B plasma by skin tests with repeated A or B plasmas and some AB plasmas are also sensitive by skin test to the purified substance. These patients are also sensitive to group A or B plasma or AB plasma administered by the intravenous route. They have reactions following the intravenous administration of the purified A and B substances. As control measures, all plasmas are tested for sterility. Some have been divided into two parts, part going to a sensitive individual with a resulting reaction and part to a nonsensitive individual of the same blood group without a reaction. Individuals sensitive to one group specific substance do not necessarily show sensitivity to the other group specific substance, although we have one instance of a patient being sensitive to both.

Patients displaying sensitivity do not necessarily have an allergic history. There does not seem to be any correlation between the amount of antibody present in the recipient and his sensitivity to the factors. However, only those individuals who are in the blood group in which the specific antibody is found corresponding to the specific A or B substance show sensitivity. Not all individuals show this sensitivity even if they have the antibody.

Are the reactions described as being due to the A and B factors important? Are they severe enough to be significant? In most of the cases, the reactions were severe enough to cause great discomfort to the patients. In all our cases we neglected to wait for a natural outcome of the reaction, since we have found that adrenalin relieves the symptoms. It is difficult to say whether any would have proved fatal without the adrenalin. We were not justified in withholding the adrenalin to find out. The severity of any reaction depends upon the reacting material and on the condition of the patient. A reaction which would not disturb a normal individual might kill a patient in a precarious condition.

A case which illustrates the practical importance of the phenomenon follows:

A. J., a woman, age 56, blood group O, had a cholecystectomy for chronic cholecystitis on November 19, 1942. Following the injection of the spinal anesthetic (10 mg. of nupercaine between L_1 and L_2), the blood pressure fell from 170/90 to 100/60. The pulse, however, remained between 70 to 80 per minute. She was given 30 mg. of ephedrine and 0.2 c.c. of neosynephrin intravenously without elevation of the blood pressure. The spinal anesthesia was supplemented with cyclopropane. For the first hour of the operation, the blood pressure remained at about 100/60 and the pulse between 70 to 80 per minute. When the blood pressure failed to rise, the patient was given 500 c.c. of pooled equal parts of A and B plasma intravenously. Ten minutes after the plasma was started, the blood pressure fell from 100/60 to 80/40 and the pulse rose from 80 to 110 per minute. It was then noticed that the patient had developed a generalized giant urticaria. The plasma was stopped, the patient was given 500 c.c. of 5 per cent glucose in saline and 3 c.c. of metrazol intravenously

with elevation of the blood pressure to 110/60 and a fall in the pulse rate to 90 per minute. The skin test to the administered plasma was strongly positive (on the operating table). Subsequently the patient was skin tested and found positive for numerous B plasmas and the purified substance. Skin tests and intravenous administration of type A plasma were without incident.

This case indicates that reactions occur under anesthesia and that they may be serious if unrecognized.

Following the recognition of the factors responsible for some plasma reactions, we were interested in the effect of pooling on the production of these reactions. Although our experience with pooled serum or plasma in cases sensitive to the A and B substances is less extensive than with group specific plasma, we have some results which are indicative. In one case previously reported,[26] the patient was sensitive to both the A and B substances. Skin tests to plasma pooled in the ration of 5:1, 2:1, and 1:1 (A:B plasma) were positive. This confirmed previous observations on other patients.[27] This patient developed a reaction to a transfusion with A plasma but not with O plasma. We then obtained a batch of pooled serum from the Human Serum Center at the University of Minnesota. The pool contained 8 group O, 8 group A, 3 group B, and 1 group AB sera. It gave a positive skin test, and after 10 c.c. had been given intravenously, the patient developed a generalized erythema, epiphora, dyspnea, rapid pulse, and a fall in blood pressure. The same serum pool gave no reaction in a nonsensitive individual.

The effect of pooling on the response to administration of the plasma in sensitive individuals was studied in a case previously reported. In this patient, a reaction occurred following transfusion of a pooled specimen of equal parts of A and B plasma, which also gave a positive skin test. The patient was only sensitive to the B plasma.

It may be that patients sensitive to a single factor may not show as marked a reaction to pooled plasma or serum due to the dilution of the A and B substances. Where the patient is sensitive to both factors, dilution would play less of a role. Neutralization of the A and B factors, in the proportions tried, does not occur in pooled specimens of plasma or serum; hence pooling is not a sure method of preventing reactions due to these substances.

REACTIONS DUE TO FACTORS OTHER THAN THE A AND B SUBSTANCES

We have evidence that reactions may occur because of factors other than the A and B substances. We have reported three cases of such reactions previously.[26] We feel that the following may be the cause of reactions when the A and B substances are not involved:

1. The presence of allergens in the plasma or serum.[28]
2. The presence of reagins in the plasma or serum.[29, 30]
3. The presence of pyrogens.
4. The presence of immunologic factors as yet unknown.

To illustrate transfusion reactions due to factors other than the A and B substances, we present the following cases observed at the University Hospitals in Minneapolis:

Case 1.—F. C., a woman aged 44, of blood group O, had lower and middle lobe lobectomy of the right lung for a bronchial adenoma on 12/11/42. During the operation, she received 400 c.c. of equal parts of A and B plasma and 300 c.c. of A plasma without reaction. On the second post-operative day, five minutes after 200 c.c. of type A plasma had run in, she developed a severe chill lasting twenty minutes with an elevation of temperature from 99 to 101° F. The skin test with the plasma which gave the reaction was positive, but tests with other plasmas, both A and B and the purified A and B substances, were negative. On 12/14/42, she received 200 c.c. of type A plasma without reaction.

Case 2.—V. E., an 18-year-old male, had a right herniorrhaphy on 11/12/42. He was given 500 c.c. of type AB plasma on 11/19/42, 500 c.c. of type B on 11/20/42, 500 c.c. of equal parts of groups A and AB on 11/21/42, 500 c.c. of equal parts of groups A and B on 11/23/42. Approximately one hour after the latter plasma had been given, the patient developed a chill and a fever of 101.6° F. The fever persisted for twelve hours. Skin tests to all plasmas were negative.

Case 3.—D. W., male, aged 44, group O, had a partial colectomy on 11/4/42 and an enterolysis and enterostomy on 11/10/42. During the postoperative period following the second operation, he received 200 c.c. of type O plasma and a similar amount of the same type of O plasma on 11/20/42. On 11/21/42, approximately twenty minutes after receiving 200 c.c. of type O plasma, the patient developed a moderately severe chill and fever of 102° F. The patient was not skin tested with the reaction-producing plasma, but subsequent tests with other group O plasmas gave negative results.

Case 4.—C. G., a 62-year-old male of blood group A had a partial colon resection for carcinoma of the sigmoid colon on 12/14/42. On 12/16/42, he received 200 mg. of equal parts of A and B plasma and 400 c.c. of type A plasma without reactions. On 12/18/42 he developed severe itching and urticaria after receiving 200 c.c. type A plasma. The skin test with this plasma was positive. On 12/19/42, after receiving 200 c.c. of type A plasma, he developed a moderately severe chill and fever of 103° F. The blood pressure fell from 114/70 to 100/70 and the pulse rose from 90 to 120 per minute; the respiration rate increased from 24 to 32 per minute. The skin test to this plasma was also positive. On 12/20/42 and on 12/23/42, he received 200 c.c. of type B plasma without reactions. On each of these occasions, the skin test was negative.

It would be difficult to assign a definite cause to these reactions, but the positive skin test would be more likely to occur associated with an allergic than with a pyrogenic type of reaction. It is certain, however, that these last cases were not due to the A or B factors. We recognize the tendency on the part of clinicians to either overlook reactions or to find them "where they are not." We cite the following case to illustrate a possible false reaction and to demonstrate our awareness of the possibility.

Case 5.—C. C., 70-year-old male, group O, was acutely ill with pyonephrosis. He had daily chills and fever ranging from 100 to 103° F. On 12/10/42 while receiving 200 c.c. of type A plasma, he again developed chills and fever at 103° F. The skin test with the administered plasma was negative. This onset of the chills and fever while plasma was being given was probably coincidental with the daily chills and fever this patient had shown previously. This conclusion was strengthened, although not necessarily proved, by the negative skin test.

CONCLUSION

We have demonstrated what we consider to be important transfusion reactions due to the A and B factors in both plasma and serum. We have also observed other reactions due to factors in plasma other than the A and B substances. We have found the skin test with the plasma a good method of indicating sensitivity to the plasma in many of the cases and an aid in preventing transfusion reactions.

REFERENCES

1. Strumia, M. M., McGraw, J. J., and Reichel, J.: The Preparation and Preservation of Human Plasma, Am. J. Clin. Path. 11: 175, 1941.
2. Strumia, M. M., Wagner, J. A., Monaghan, J. F.: The Use of Citrated Plasma in the Treatment of Secondary Shock, J. A. M. A. 114: 1337, 1940.
3. McGuiness, A. C., Stokes, Jr., J. Mudd, S.: Clinical Uses of Human Serums Preserved by the Lyophile Process, J. Clin. Investigation 16: 185, 1937.
4. Ravdin, I. S.: Report of the Blood Transfusion Association, January, 1941.
5. Meakins, J. C.: Ibid., January, 1941.
6. Levinson, S. O., Rubovits, Jr., F. E., Necheles, H.: Human Serum Transfusions, J. A. M. A. 115: 1163, 1940.
7. Mellanby, E.: Report of the Blood Transfusion Association, January, 1941.
8. Self, E. B., Scudder, J.: The Therapeutic Use of Serum and Plasma, Liquid and Dried. Blood Substitutes and Blood Transfusions, Springfield, Ill., 1942, Charles C Thomas Company, p. 377.
9. Stephenson, C. S., Cushing, E. H., and Newhouser, L. R.: The Present Status of Blood Substitutes in the United States Navy, Ibid., p. 221.
10. Elliott, J., Busby, G. F., and Tatum, W. L.: Some Factors and Observations on Preparation and Preservation of Dilute Plasma, J. A. M. A. 115: 1006, 1940.
11. Polayes, S. H., and Squillace, J. A.: Near Fatal Reaction to Transfusion With Dried Human Plasma Solutions, J. A. M. A. 118: 1050, 1942.
12. Levine, M., and State, D.: A and B Substances as a Cause of Reactions Following Human Plasma Transfusions, J. A. M. A. 120: 275, 1942.
13. Mahoney, E. B., Kingsley, H. D., Howland, J. E.: The Therapeutic Value of Preserved Blood Plasma. Blood Substitutes, etc., Springfield, Ill., 1942, Charles C Thomas Company, p. 345.
14. Strumia, M. M., McGraw, J. J.: Blood Plasma, J. A. M. A. 118: 427, 1942.
15. Bowditch, H. P.: Arbeit. a.d. physiol 6: 139, 1871, Anstalt zu Leipzig.
16. Moldovan, J.: Ueber die Wirkung intravaskularer Injektionen frischen, defibrinierten Blutes, Deutsche med. Wchnschr. 2: 2422, 1910.
17. Janeway, T. C., Richardson, H. B., and Park, E. A.: Experiments on the Vasoconstrictor Action of Blood Serum, Arch. Int. Med. 21: 565, 1918.
18. Amberson, W. R.: Blood Substitutes, Biological Reviews of the Cambridge Phil. Soc. 12: 48, 1937.
19. Levine, M.: Blood Bank Problems, Bull. Staff Meeting, Hospitals of the University of Minnesota 13: 154, 1942.
20. Moss, W. L.: Studien über Isoagglutinine und Isohemolysine, Folia serolog., 267, 1910.
21. Schiff, F.: Zur Kenntnis blutgruppenspezifischer Antigene and Antikorper, Klin. Wchnschr. 3: 679, 1924.
22. Levinson, S. O., and Cronheim, A.: Suppression of Iso-Agglutinins and the Significance of This Phenomenon in Serum, J. A. M. A. 114: 455, 1940.
23. Lubinski, H. H.: The Inhibitory Effect of Human Serum Upon the Iso-Agglutination of Red Cells, J. Immunol. 44: 95, 1942.
24. Aubert, E. F., Boorman, K. E., Dodd, B. E.: The Agglutinin Inhibiting Substance in Human Serum, J. Path. & Bact. 54: 89, 1942.
25. Witebsky, E., Klendshoj, N., Swanson, P.: Reduction or Elimination of the Anti A Antibody in O Blood by Means of the Addition of the "A" Specific Substance, J. Infect. Dis. 67: 188, 1940.
26. Levine, M., and State D.: Plasma and Serum Transfusion Reactions Due to Factors Other Than the A and B Substances. In press.
27. Levine, M., and State D.: Skin Sensitivity to Human Plasma, Science 96: 68, 1942.
28. Duke, W. W., and Stofer, D. D.: A Severe Case of Allergy Due to Fish Glue, Med. Clin. North America 7: 1253, 1924.
29. Holder, A. G., and Diefenbach, W. E.: Urticaria—Its Passive Transmission by Blood Transfusion, California and West. Med. 37: 387, 1932.
30. Garver, W. P.: The Transference of Reagins in Blood Transfusions, J. Allergy 11: 32, 1939.

THE EFFECT OF SULFONAMIDE COMPOUNDS ON ANTIBODY RESPONSE TO STAPHYLOCOCCUS TOXOID*

JAMES B. WEAVER, M.D., AND MARY WHELAN TYLER, M.D., KANSAS CITY, KAN.

THIS study was undertaken to determine the effect of sulfanilamide and related compounds on the production of antibodies in response to staphylococcus toxoid injections. Only one paper on this subject was found in a review of the literature. Richou and Rastigar[1] found that there is apparently no inhibition of response in antibody development following simultaneous injections of sulfanilamide and staphylococcus anatoxin. They used two groups of five rabbits each. To one group they gave three injections of 0.5 to 3 c.c. of staphylococcus anatoxin at five-day intervals. To the second group they gave anatoxin as to Group 1 plus two intravenous injections at several hour intervals of 3 c.c. of a solution of sulfanilamide containing 1 gram in 150 c.c. saline (0.2 grain at each injection). The dose of the drug was relatively small and the length of the experimental period was short. Increasing the amount of drug given to levels comparable to that given therapeutically and continuing to administer it daily for a sufficiently long period should give a truer picture. Consequently the following plan of procedure was carried out.

White rabbits weighing between five and six pounds each were used. Staphylococcus toxoid and drug injections were begun simultaneously. Toxoid was injected subcutaneously beginning with 0.1 c.c. and increasing by 0.1 c.c. at each injection until 1 c.c. was given at a single injection. Injections were made three times a week. The soluble salts of the drugs (sulfanilamide, sulfapyridine, and sulfathiazole) were used to insure constant dosage, and as nearly as possible complete utilization. Injections were made daily throughout the life of this experiment, a period of eight to nine weeks. An attempt was made to keep the daily dose of the drugs within therapeutic limits, because we were chiefly interested in the effects produced by these drugs in the amounts usually prescribed clinically and not in the effect produced by pushing the dose to toxic levels. A control group of nine rabbits received only toxoid. Antihemolysin titers, total leucocyte counts, and hemoglobin determinations were made twice a week.

NEOPRONTOSIL

Six c.c. of neoprontosil were injected intramuscularly daily to each of seven rabbits, making an average dose of 120 mg. per kilo of body weight, which would be equivalent to 8.4 grams or 140 grains daily to a 70-kilo man. Hemoglobin levels fluctuated slightly but not more than is observed in control

*From the Hixon Laboratories of the University of Kansas School of Medicine and financed by federal funds obtained through the Kansas State Board of Health.

Received for publication, May 17, 1943.

animals living under the same conditions. Total leucocyte counts were not affected, and the antihemolysin response to toxoid injections occurred at approximately the same time after the initial injection and reached a level somewhat higher, on the average, than the controls. (Chart 1.)

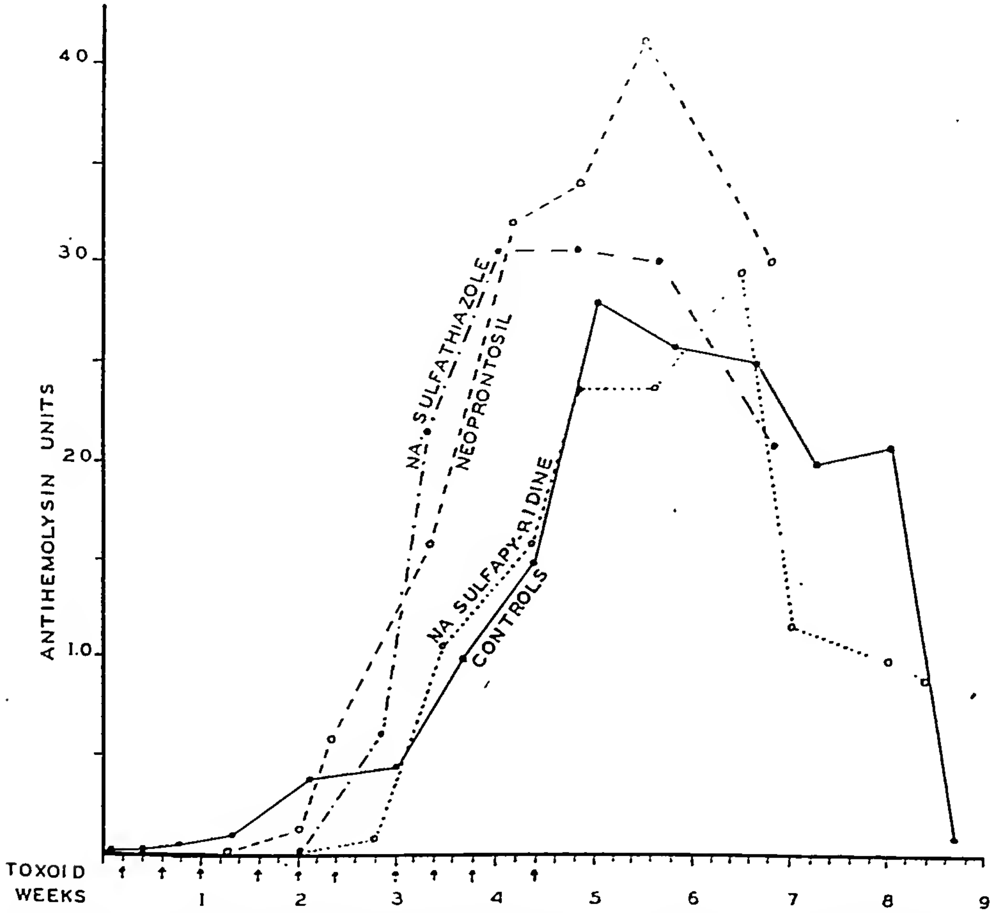


Chart 1.—Composite curves of serum antihemolysin response following staphylococcus toxoid injections in a series of control rabbits and rabbits to which neoprontosil, sodium sulfathiazole and sodium sulfapyradine were given.

SODIUM SULFATHIAZOLE*

Sodium sulfathiazole was made up in 5 per cent solution and given in the amount of 60 mg. per kilo intravenously to six rabbits. The results obtained were similar to those observed during the administration of neoprontosil. No marked fall of hemoglobin concentration was observed, and the total leucocyte count was not markedly reduced, except in two instances when counts of less than 5,000 were obtained. This, however, was only temporary, for normal counts were obtained on the following day, and no subsequent reduction was observed. Chart 1 shows that the average serum antihemolysin curve is similar to the control, except that a slightly more rapid response occurred with a slightly higher level. The concentration of the drug in the blood reached levels of from 1.3 to 5 mg. per 100 c.c.

*Kindly supplied by Dr. Newcomer of Squibbs Institute.

SODIUM SULFAPYRIDINE

A 5 per cent solution of sodium sulfapyridine was used for intravenous injections. Approximately 100 mg. per kilo were given to each of five rabbits daily. Chart 1 shows the antihemolysin curve and its similarity to the controls. The drug reached concentrations of from 1.5 to 3 mg. per 100 c.c. in the blood. Three rabbits were given an average daily injection of 250 mg. per kilo. This was done to see if doubling the therapeutic dose would affect the antibody response in any way. There was no detrimental effect, since the response to toxoid injections was as rapid and the height of the antihemolysin curve was as great as that obtained in the control rabbits. Determinations of sulfapyridine in the blood gave values of 2.5 to 9 mg. per 100 c.c. Hemoglobin concentrations and the total leucocyte counts were not affected by either dosage.

COMMENT

Sodium sulfapyridine, sodium sulfathiazole, and neoprontosil, when given to rabbits in doses which correspond to the average therapeutic dose or higher, have no effect on hemoglobin concentration, leucocyte count, or antibody response to staphylococcus toxoid injections. The antihemolysin titers became positive as soon, and in some cases sooner, in control rabbits and reached correspondingly high levels. In general, the same type of curve was obtained, the fall in titer occurring at approximately the same time after the last toxoid injection.

REFERENCE

Richou, R., and Rastigar, R.: Les injections de sulfamide, faites simultanement a des injections d'anatoxine staphylococcique, ne semblent pas influencer chez le lapin, le developpement de l'antitoxine specifique, Comp. rend. Soc. de Biol. 131: 855, 1939.

A NOTE ON CYSTS AND ABSCESSES INDUCED IN THE RAT BY THE INJECTION OF OILS*

FREDERICK E. EMERY, PH.D., AND CHARLES S. MATTHEWS, M.A.
BUFFALO, N. Y.

RECENTLY WE showed that peanut oil and sesame oil formed cysts when injected into the muscle of rats. Following this report,[1] several inquiries were received from persons interested in the use of oils as vehicles for hormones and other drugs. Therefore, additional experiments were performed, and in this paper we present data pertaining to the formation of cysts and abscesses following intramuscular injections of mazola, olive, cotton seed, sweet almond, sesame, and peanut oil.

METHODS

The procedure was the same for all oils and was performed by giving a single intramuscular injection of 1.0 c.c. of oil in the hind leg muscles of the rats. These rats were killed from two days to one year after the start of the experiment, and the legs were carefully examined for oil, hemorrhages, durability of cyst walls, and abscesses.

RESULTS

Mazola Oil.—The data show that this oil readily forms cysts. These cysts have firm, somewhat transparent walls and contain clear oil. They persist in the muscles for long periods of time and were found at autopsies performed as long as twelve months after the oil was injected (Fig. 1). Not one abscess has been found in more than fifty experiments with mazola oil (Table I).

TABLE I

AUTOPSY DATA TAKEN AT VARYING PERIODS OF TIME FROM A FEW DAYS TO ONE YEAR AFTER AN INTRAMUSCULAR INJECTION OF 1.0 C.C. OF OIL

NUMBER OF EXPERIMENTS	OILS	CONDITION OF OIL	CONDITION OF CYSTS			
			ABSCESSES	PER CENT	CYST WALL PRESENT	CYST WALL ABSENT
39	Mazola	Often yellow	None	--	37	2
50	Olive	Clear	None	--	48	2
46	Cotton seed	Clear or yellow	8	17.4	40	6
108	Sweet almond	Clear or necrotic	43	39.8	97	11
30	Sesame	Clear	None	--	30	None
35	Peanut	Clear	None	--	33	2

Olive Oil.—Cysts were nearly always present after intramuscular injections of olive oil. The cyst wall was thin and more easily broken than was the case with any other oils studied. Yet even these cysts may remain in the muscles for many months (Fig. 1). Oil taken from the cysts was almost always clear

*From the Department of Physiology University of Buffalo.
Received for publication, May 31, 1943.

and free from necrotic foci. Gross abscesses were not found. These facts give olive oil a high rating for intramuscular injection.

Cotton Seed Oil.—Intramuscular injections of cotton seed oil were usually followed by oil cysts. In six out of forty-six experiments cysts were not found. These negative cases are more frequent than those recorded for mazola and olive oil (Table I). The cyst walls appeared to be firmer than those formed after injection of mazola, olive, and peanut oils. Oil taken from the cysts, at intervals, during the first four months, was clear and showed no evidence of infection. The eight abscesses found (Table I) were all found in autopsies performed five to twelve months after the injections were given. Some of these were necrotic areas buoyed by the oil, while others were firm, yellow, necrotic areas from which oil could not be obtained. Since the abscesses were found only after the lapse of considerable time, it seems likely that the necrosis was not due to the irritation of the oil originally injected, but more probably developed after the oil had changed in composition and, hence, had become more irritating to the tissues. These abscesses were never found in the acute inflammatory condition as described below for sweet almond oil.

Sweet Almond Oil.—Table I shows that cysts were formed in nearly all rats injected intramuscularly with sweet almond oil. The cyst walls were well developed, semiopaque in color, and in about half of the cases so durable that the entire cyst could be removed unbroken from the muscles. In these cases the oil was usually clear. Abscesses in various stages of development were found in nearly half of the rats (Table I). These varied from spots floating in the oil to large inflammatory masses weighing as much as ten grams. When both hind legs were injected on the same day, the bilateral swelling of the legs was so severe that a few of the rats thus treated died within five days. Fig. 1 shows various sizes of the abscesses. Sometimes within a few days following the injection of sweet almond oil, the whole area of the leg was inflammatory, the oil floating in tissue fluid tinted with hemoglobin. Within a week or ten days the swelling subsided, and autopsies showed the oil largely or entirely gone and the capsule containing pus in varying amounts. Thus the picture of sweet almond oil after intramuscular injection is not gratifying.

Sesame Oil.—After intramuscular injections of sesame oil, cysts were readily formed, thus confirming previous reports.[1, 2] Additional experiments carried out during the present investigation serve as controls for the other oils, especially in regard to thickness and toughness of the capsule wall and a tendency for abscess formation. The wall developed around sesame oil is quite tough, and the entire cyst can be dissected readily from adjacent tissues without breaking. This is especially true of cysts that have been in the muscles for several months, but even in earlier stages of development the cysts formed around sesame oil are more durable than any of the other cysts studied. Abscess formation is rare, although flocculent white specks involving part or nearly all of the oil are sometimes seen.

Peanut Oil.—The injection of peanut oil intramuscularly was followed by the formation of cysts. The cyst wall is not as thick or durable as those induced by sesame oil. The oil found in the cysts is fairly abundant in amount and clear. Abscesses have not been seen in any of the rats at autopsy. This

oil seems to irritate the tissues very little and, therefore, compares favorably to mazola and olive oils; so far as we can tell, it differs from sesame oil only in the thickness and durablity of the cyst wall.

DISCUSSION

The results described make one wonder if oil should be injected intramuscularly;* certainly in rats it is obvious that sweet almond oil is very irritating to the tissues, and acute inflammation and even death may result. Cotton seed oil is less irritating, and the injected area as a rule does not become swollen; in only a few rats were abscesses formed (Table I).

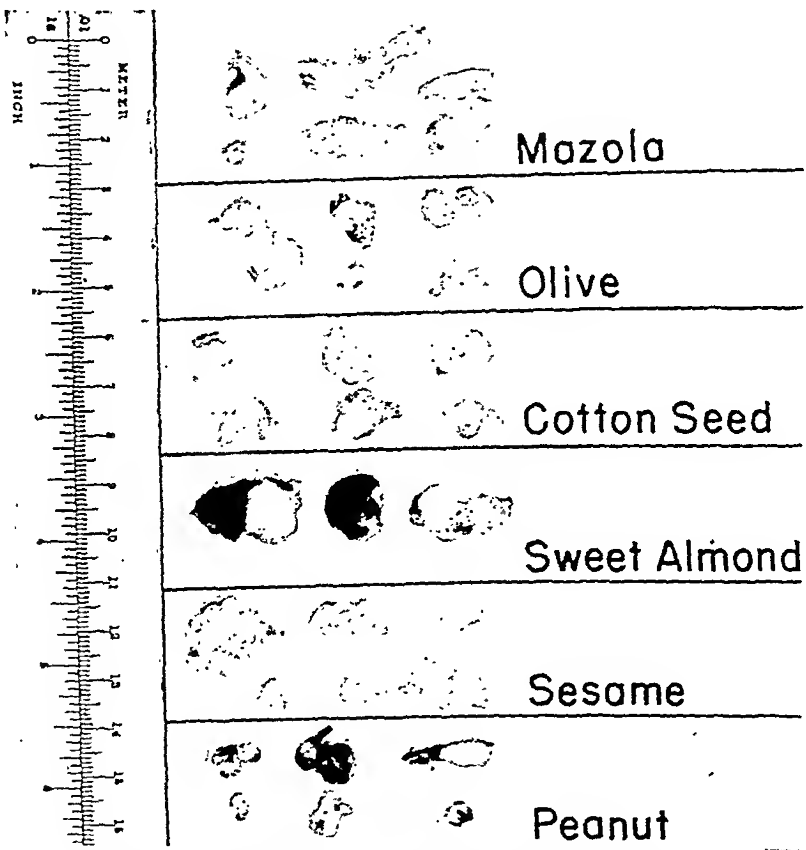


Fig. 1.—Cysts containing oil dissected from the muscles of rats after being in the muscles for varying periods up to one year.

Sesame oil induces thick and durable cystic walls which usually contain clear oil, thus confirming previous reports.[1, 2] These differences place sesame oil in preference to the sweet almond and cotton seed oil for intramuscular injections. As judged by the thickness of the capsule, mazola, olive, and

*Several letters received in regard to our first report contained inquiries concerning oil cysts in humans. Although our data are from rats, the following comments may be of value: Usually autopsy of the leg muscles is not performed. It seems likely that oil cysts would develop, but due to the small size may not be noticed by the patient. Some physicians have told us of complaints by the patient following intramuscular injections of *sesame oil*. An interne at a local hospital started to give an intramuscular injection of oil, and as a precaution he inserted the needle and aspirated. He withdrew 4 c.c. from an oil cyst formed in the muscles after previous injections. When asked what he did with the oil, he replied, "I injected it all back again."

peanut oils are less irritating than sesame oil; yet it is difficult to place one of these as first choice for intramuscular injection. In our experience all four failed to induce gross inflammatory reactions in the muscles, but like all the other oils used in this study they form oil cysts which remain in the muscles for several months.

When any one of these six oils was injected subcutaneously, the globule readily disseminated, cysts were infrequently seen at autopsy, and abscesses were never found.

Usually the injection was not carried out with antiseptic technique. Since abscesses so readily developed after the injection of sweet almond oil, careful sterilization of the oil, syringe, and needle and application of alcohol to the injected area was followed in many subsequent experiments. This careful technique had no bearing on the incidence of infection.

SUMMARY

Intramuscular injections of mazola, olive, cotton seed, sweet almond, sesame, and peanut oils were given in the hind leg of rats. A tissue capsule developed around the oil within a few days, forming an oil cyst which may remain in the muscles for at least a year. Abscesses developed in nearly half of the rats injected with sweet almond oil and in a few of those injected with cotton seed oil. Sesame oil formed a more durable cyst wall than mazola, olive, and peanut oils, but all four are less irritating than sweet almond and cotton seed oil.

REFERENCES

1. Emery, F. E., Matthews, C. S., and Schwabe, E. L.: The Absorption of Stilbestrol and Theelin From Cysts of Sesame and Peanut Oils, J. Lab. & Clin. Med. 27: 622, 1942.
2. Deanesly, R., and Parkes, A. S.: Note on Subcutaneous Absorption of Oils by Rats and Mice, With Special Reference to the Assay of Oestrin, J. Physiol. 78: 155, 1933.

ACUTE DISSECTING AORTIC ANEURYSM: TWO CASES WITH AN ANTE-MORTEM DIAGNOSIS IN ONE*

S. L. ZIMMERMAN, M.D., COLUMBIA, S. C.

DISSECTING aortic aneurysms present a varied clinical syndrome, and it is unusual for the process to present a clear-cut picture suggesting an ante-mortem diagnosis. In addition, the entity is not a common one. Flaxman[1] reports an analysis of 19 cases of dissecting aortic aneurysms which were noted in 14,160 autopsies at the Cook County Hospital between 1929 and 1941, an incidence of 0.14 per cent. In none of these cases was a definite ante-mortem diagnosis reached. He reports a similar incidence at the Charity Hospital of Louisiana, at New Orleans, and the Massachusetts General Hospital.

Up until 1933, 300 cases of dissecting aortic aneurysms were analyzed by Shennan,[2] and in addition 112 more cases have been reported. Prior to 1933 a correct ante-mortem diagnosis was made in only seven cases, and since that time the ante-mortem diagnosis has been made in 25 additional cases, bringing the total to 32 (7.9 per cent) out of 431 reported dissections.[1] In those cases in which an ante-mortem diagnosis was made there were "pointing" clinical findings, such as a sudden onset of a severe, tearing pain, usually thoracic, with variable radiation, collapse, disparity in blood pressure recordings in the two upper extremities, roentgenologic changes, etc. The main pitfall in its correct diagnosis appears to have been the confusion with coronary occlusion. The absence of a typical electrocardiographic evolution of a protracted coronary insufficiency is helpful in those cases which survive the acute dissection. Weiss,[3] however, has reported a case in which the dissection invaded the coronary artery, producing a localized area of myocardial ischemia and typical electrocardiographic changes. In those cases which may be termed "recent" the problem is more difficult. Here the entire gamut of the causes of severe thoracic pain must be differentiated, if pain be a presenting feature. It is well known on the other hand that many dissections are "silent" and are picked up at the necropsy table, much to the surprise of the clinician.

A dissecting aneurysm is a disease of later life, and it is rare before the age of fifty.[4] Flaxman,[1] however, reports one case in a youth twenty-two years of age with hypertensive heart disease.

The exact mode of its production is still a matter of conjecture. It is not definitely known whether an intimal crack or an atheromatous plaque with subsequent medial dissection presents a true picture of its genesis. The other possible mechanism advanced has been the rupture of one or more of the vasa vasorum into an already degenerated media with a subsequent formation of a hematoma. Whether or not the intimal tear results from the rupture of the

*From the Cardiological Service, U. S. Veterans Adm. Facility.
Published with the permission of the Medical Director of the Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.
Received for publication, June 2, 1943.

hematoma inwardly or precedes the dissection is not definitely known. The media is usually degenerated, with the production of an inelastic and brittle tube. Cyst formation, idiopathic in origin, with medial necrosis is sometimes found.

Syphilis has often been incriminated as an etiological factor. It is now known that a great many cases show no evidence of syphilis, although the two entities are by no means mutually exclusive. The role that syphilitic mesaortitis plays, in addition to a "nonspecific" medial disease, may enhance the occurrence of a dissection.[4] Hypertension, usually severe, is present in the great majority of cases. It was present in all of the 19 autopsied cases reported by Flaxman.[1] Syphilis was proved in 3, but in conjunction with hypertensive heart disease.

The mode of exitus is usually sudden and dependent upon rupture of the newly formed channel, most often into the pericardial sac, the pleural sacs, or into the peritoneal cavity. Multiple ruptures have been reported. Congestive failure, chronic but progressive, is not an uncommon termination of those cases which are protracted in their courses.

The electrocardiographic changes are often due to a coincident coronary arteriosclerosis; thus, any variety of QRS, ST-T combinations may be seen. If there is a latent coronary insufficiency, the ensuing fall in blood pressure may occasionally result in myocardial ischemia and produce electrocardiographic patterns suggestive of an occlusion. These, however, are usually slighter in degree.[5] If rupture into the pericardial sac results in cardiac tamponade, diagnostic tracings may sometimes be obtained.

The following case is reported as an additional one, unusual in the sense that an ante-mortem diagnosis was made relatively simple by the clinical course of the patient. This largely resulted because the patient was hospitalized during the time of the dissection and was observed at the probable moment of rupture into the abdomen and pericardial sac. The second case is one in which a diagnosis was not made, and it probably is typical of the usual aortic dissection in a patient who is hospitalized shortly before death. It is probable that the dissection had taken place prior to admission and that he had ruptured on the ward.

Case Reports

J. S. H., a fifty-two-year-old white male, was admitted on November 19, 1942, with a diagnosis of hypertension and arteriosclerosis. Past history was not relevant, except that there was a history of syphilis in 1916, followed by three injections of "606." Repeated subsequent serology was negative. He was a known hypertensive, asymptomatic, until four months prior to admission, at which time he noticed exertional dyspnea, progressing to the point of dyspnea at rest and orthopnea. About the same time he first became cognizant of attacks of paroxysmal nocturnal dyspnea, which increased in frequency and severity up to the time of admission. There was no history of anginal syndrome or dependant edema.

Cardiologic examination on November 24 revealed a seriously ill, dyspneic and orthopneic male, slightly cyanotic. The carotid pulsations were exaggerated. The peripheral vessels were sclerotic. Pulses were unequal, the right having a greater amplitude than the left. Blood pressure, right arm, was 220/150; left arm, 180/150. The point of maximum intensity was felt in the fifth intercostal space well outside the midclavicular line. There was some slight increase in retromanubrial dullness. The rhythm was regular. The first sound over the mitral area was of fair quality. No thrills were palpated and no murmurs were heard. Both second sounds over the bases were accentuated. On inspiration, bilateral basal râles were

heard. The liver was not felt; there was no systemic venous engorgement, and no dependent edema was present.

Visualization of the fundi revealed blurring of both discs, many hemorrhages, and hard exudates. The retinal vessels were tortuous, irregular, and fragmented. Wassermann and Kahn tests were negative. Casual urine examinations revealed a trace of albumin and occasional red blood cells. Nonprotein nitrogen was 35 mg. per 100 c.c. Electrocardiogram taken on the day following admission revealed a regular sinus rhythm, with a rate of 95. There were changes indicating left ventricular preponderance; the P-R interval varied from .13 to .16 seconds, with some changes in the configuration of the P waves in succeeding beats. QRS complexes were slurred. The T waves were inverted in 1 and 2 and upright in 3. S-T junction was slightly depressed in the first and second leads. Lead 4-F showed diphasic QRS complexes with an inverted T wave and slight elevation of the S-T junction.

Fig. 1 (Case 1).—Photograph of heart and supracardiac portion of the aorta. Note the linear rent in the supravalvular portion of the aorta. There is no separation of the commissures and no dilatation of the aortic ring. Note the tremendous hypertrophy of the left ventricular musculature. The small markings above the tear are artefacts, due to a hemostatic forceps having been applied.

Interpretation was: 1. Myocardial damage, based on inverted T waves in leads, 1, 2, and 4, depressed ST junction in leads 1 and 2, and slurring of the QRS complexes. 2. Left ventricular preponderance. 3. Wandering pacemaker. X-ray of the chest revealed an enormously increased cardiac shadow with some hypostatic congestion in both bases. The aortic shadow was reported normal. The clinical impression was: 1. Malignant arteriolonephrosclerosis, in impending uremia. 2. Hypertensive and coronary arterio-sclerotic heart disease with cardiac enlargement, myocardial damage, and left ventricular failure. 3. Hypertensive neuroretinopathy.

Clinical Course.—The course was entirely afebrile. Respirations varied between 18 and 22; pulse rate ranged between 80 and 100. The patient was digitalized on November 25, 1942, and he showed gradual improvement, so that by November 29 there was no longer evidence of left ventricular failure. At approximately 9 P.M., December 3, he was suddenly taken with severe persistent pain beginning in the region of the suprasternal notch and radiating to the lower chest, abdomen, and down the back, associated with, and followed by, a sensation of numbness in his left leg. Nausea and vomiting were both present. This pain lasted at least three hours, in spite of narcotics. At 9 A.M., December 4, he still complained of numbness in his left leg, and of nausea and vomiting. Examination at that time revealed that he had developed a loud, rough, systolic aortic murmur, which was heard throughout the entire precordium. There was a short, softer, diastolic murmur heard over the aortic area and along the left sternal border. No thrill was palpated. There was no evidence of distention of the neck veins, and the blood pressure was as follows: Right arm, 230/100;

Fig. 2 (Case 1).—Same as depicted in Fig. 1, except that the superior leaf has been elevated to better visualize the tear. See text.

left arm, 150/130. The left dorsalis pedis artery had a markedly diminished pulsation. A tentative diagnosis of acute dissecting aortic aneurysm was made. Beside roentgenogram revealed tremendous aortic and cardiac shadows, but no comparison with the teleoroentgenogram was possible, in view of the differences in technique. It must be stated, however, that the aorta appeared definitely wider than one would expect, were only such differences considered. Electrocardiogram revealed changes in the ST and T contours, the most important of which were the development of upright T waves in the fourth lead and a diphasic T wave in the second lead, as compared to previously inverted T waves. ST junctions were de-

pressed in the first and second leads, and were within normal limits in the fourth lead. The rhythm was regular, rate 150. There were no changes indicative of an acute cardiac tamponade, and there were no changes reported suggesting a diagnosis of acute coronary occlusion. At 4:30 P.M. it was difficult to feel either dorsalis pedis artery. The right posterior tibial artery pulsated normally, but the left could not be felt. He merged into rather rapid congestive failure; pulses were very unequal in amplitude; blood pressure, right arm was 260/110, and could not be obtained by the auscultatory method in the left arm; however, by palpation, the systolic pressure approximated 140 mm. of mercury. At 9 A.M. the following morning he appeared terminal and expired at 9:12. His abdomen became rigid a few minutes prior to his exitus. The final diagnosis was: 1. Malignant arteriolo-nephrosclerosis.

Fig. 3 (Case 1).—Attention is invited to the severe atheromatous changes in the entire thoracic and abdominal aorta. In the lateral view of this it can be seen that the layers have been separated by blood, but the exact point of rupture into the abdominal cavity was not found.

2. Acute dissecting aortic aneurysm, with rupture into the abdomen and pericardial sac. 3. Hypertensive and coronary arteriosclerotic heart disease. Myocardial damage, cardiac enlargement, congestive failure. 4. Hypertensive neuroretinopathy.

POST-MORTEM EXAMINATION, CASE 1

On opening the abdomen, there was a gush of fresh blood which was not clotted, estimated at approximately 750 c.c. The peritoneal surfaces showed little, if any, reaction. On opening the chest, both lungs were found to be normal in appearance. There was no fluid in the pleural sacs. Both lungs had a normal consistency. The right weighed 500 grams, and the left, 600 grams. The cut surface presented no areas of atelectasis or consolidation. The pericardial sac was tensely distended with approximately 500 c.c. of fluid and clotted blood. The serosal surfaces were smooth and glistening. The heart was strikingly enlarged and weighed 800 grams. There was a bluish discoloration of the epicardium posteriorly around the base of the aorta and pulmonary artery. A small rent, 6 mm. in length, extending through the epicardium and the adventitia of the aorta, was noted 2 cm. above the level of the aortic cusps. This could be followed into a cavity separating the adventitia and the media of the aorta.

Fig. 4 (Case 1).—Section through the lower portion of the abdominal aorta. The dark black areas depict clotted blood which has dissected between the media and the adventitia, not quite encircling the aorta.

The wall of the right ventricle was moderately hypertrophied. The bluish discoloration overlying the base of the pulmonary artery was due to a collection of blood, apparently between the media and the adventitia of the pulmonary artery from its origin to a point 3 cm. above the valve cusps. The wall of the left ventricle was markedly hypertrophied, averaging 23 mm. in thickness. The cavity was moderately dilated. The aortic valve cusps were thin and delicate. There was no fusion or widening of the commissures. The ostia of the coronary arteries were patent. The entire aorta was markedly sclerotic, stiff, and inelastic. There was a linear tear in the intima of the aorta at a level 5 mm. above the valve commissures and extending transversely across the right posterior and most of the left posterior valve. This measured 4 cm. in length. The edges were slightly ragged. This tear extended through the intima and media and opened into a complex cavity. This cavity extended, as already described, through a rent in the base of the aorta into the pericardium. It also extended the entire length of the aorta, creating a potential space between the ad-

ventitia and media which in some areas contained clotted blood. It did not completely surround the aorta, but passed about halfway around the circumference at most levels. The arteriosclerosis of the aorta was greatest in the descending portion, the intima of the ascending aorta being almost smooth. There were no tree bark wrinklings of the intima or other luetic stigmata. The exact site of perforation into the abdomen could not be ascertained, but there were no prominent ecchymoses in the periaortic fat at any point. The coronary arteries were diffusely thick-walled, and the lumen was narrowed to about ⅓ of normal. There were no areas of occlusion, however, and there was no dissection of the walls. The myocardium was red-brown and firm, and there were no areas of softening or fibrosis.

MICROSCOPIC EXAMINATION

Heart.—There was a marked hypertrophy of the muscle fibers which had large, irregular, and bizarre nuclei. In a few small areas there was a focal degeneration of occasional muscle fibers. No histologic evidence was seen of inflammatory reaction to the hemopericardium.

Aorta.—The intima was generally thickened. There were several atheromata of typical appearance, varying from the cellular phase to acellular debris with clefts where the cholesterol had been dissolved. The media was divided approximately in the middle by the dissection. In the resulting space was free blood and also adherent platelet-fibrin-leucocyte thrombi. The parallel elastic fibers of the media were intact, except for the two to three fibers immediately adjacent to the dissection where they were occasionally necrotic and often disorganized by the seepage of blood. In a few areas there was a sparse leucocytic infiltration. The line of tear was parallel to the elastic fibers. In the outer layer of the media were occasional small blood vessels and fibrotic scars, both at right angles to and parallel to the elastic lamelae. In the adventitia was a perivascular collection of small round cells, some of moderate magnitude. These inflammatory changes were not diagnostic of a syphilitic meso-aortitis.

Anatomic Diagnosis.—1. Dissecting aneurysm of the aorta with rupture into the abdomen and pericardium. 2. Marked hypertrophy of the left ventricle. 3. Arteriosclerosis of the aorta, severe. 4. Coronary arteriosclerosis. 5. Arteriosclerosis of the kidneys.

CASE NO. 2

E. W. B., a 47-year-old white male, was admitted at 4 P.M., January 13, 1943. In his left lateral chest he had had a severe attack of pain, which radiated to his substernal region on the evening of January 11, 1943. The pain was oppressive in nature and later radiated to the epigastrium; following this he became very weak and broke out into a profuse perspiration. The pain also radiated to both shoulders, but not down either arm. He vomited one and one-half hours later. At time of admission the only residual complaint was some pain in his epigastrium. He was a known hypertensive for many years, but had no complaints referable to his cardiovascular system. There was no history of rheumatic fever or syphilis.

Cardiologic examination, January 14, 1943, revealed a 47-year-old white male, propped up in bed, dyspneic and cyanotic, appearing acutely ill. There was a regular sinus tachycardia. Point of maximal intensity was in the fifth intercostal space at the midclavicular line. The apical impulse, however, was heaving and forceful and gave the impression of a markedly hypertrophied left ventricle. The sounds were booming with an accentuated A-2. There were no thrills palpated and no murmurs heard. There was no evidence of failure. Blood pressure was 240/140. The radial vessels were thickened. Electrocardiogram revealed inverted T waves in the second and third leads and the presence of left axis deviation. There was no evidence of an acute myocardial infarction.

The white blood count was 13,600, with 85 per cent polymorphonuclears and 15 per cent lymphocytes. Nonprotein nitrogen was 35. The urine was not remarkable. Wassermann and Kahn tests were negative. The temperature was 100 degrees.

A diagnosis of hypertensive and coronary arteriosclerotic heart disease, with cardiac enlargement, was made, and in spite of the elevated blood pressure, the probability of a coronary thrombosis was borne in mind. A short time following the above examination the patient expired, quietly and rapidly. Just prior to death the cardiac sounds were not discernible, and the possibility of a ruptured ventricle resulting in an acute cardiac tamponade was considered.

POST-MORTEM EXAMINATION, CASE 2

There was an extravasation of bluish red blood into the retroperitoneal tissues. This extravasation was localized, primarily, on the posterior aspect of the aorta, but also extended down along the right iliac artery almost to the pelvic outlet, around the right lateral surface of the bladder and into the base of the mesentery.

Chest.—There was no fluid nor were there adhesions in the right pleural cavity. In the left pleural cavity there were about 2000 c.c. of fresh blood containing very large fresh clots. The heart was moderately enlarged and weighed 500 Gm. There was an ecchymosis into the adventitia of the pulmonary artery which will be described subsequently. The wall of the left ventricle was markedly thickened, averaging 20 mm. The cavity was moderately dilated. The aortic valve cusps were thin and delicate but contained a few atherosclerotic plaques. There was a moderate arteriosclerosis within the sinuses of Valsalva, which resulted in a moderate fusion at the commissures of all the cusps. The ostia of the coronary arteries were widely patent. The coronary arteries were the seat of widespread and severe arteriosclerosis. There was no marked narrowing of the lumina in the first two centimeters. Beyond the first two centimeters the lumina of all major vessels were markedly narrowed at most levels to a mere pin point. The myocardium was red-brown and firm, and there were no areas of fibrosis noted. The ascending aorta and the arch were elastic, and the intima was almost free of atherosclerosis. Just beyond the opening of the left subclavian artery there was a long oblique linear rent extending through the intima and media. It was 4.5 cm. in length and occurred in a section of the aorta which was free of calcified plaques or other grossly evident abnormalities. From this point down, there was a cavity between the media and adventitia which almost completely encircled the aorta to the level of the origin of the inferior mesenteric artery. At this point, there was another transverse linear rent in the intima 1.5 cm. in length and communicating with the cavity. Below this, the dissection continued down the right side of the aorta and the right iliac artery. The left half of the aorta was not dissected at this level. Within this cavity was a large quantity of freshly clotted blood about 2 cm. in thickness at the level of the upper tear, and diminishing to a few mm. below. The intima of this portion of the aorta contained a considerable number of superficial yellow plaques, a few of which were calcified. There was no tree bark wrinkling of the intima. There was a moderate loss of elasticity of the wall. There was a marked ecchymosis into the adventitia; this was most marked in the thoracic portion of the descending aorta. It also spread upwards, though mild in degree, over the arch of the aorta, on the anterior surface of the aorta and pulmonary arteries to their bases, and into the parietal layer of the pericardium for about 6 cm. from its attachment. There was no dissection between the media and adventitia above the proximal tear. The aorta measured 6 cm. in diameter in the ascending portion and tapered down to 5.5 cm. in the descending portion. There was no constriction at any point or coarctation. The point of rupture of the adventitial tissues into the left pleural cavity could not be determined.

MICROSCOPIC EXAMINATION

Aorta.—The intima was essentially normal. The inner half of the media was composed of the usual parallel lamellae of elastic tissue. The dissection separated the outer quarter from the inner three-quarters of the media. In the space created was an adherent thrombus composed of fibrin and red blood cells with a considerable infiltration of polymorphonuclears. The inner segment of the media subjacent to the thrombus stained poorly, and the nuclei of the elastic and connective tissue fibers appeared to have dropped out. This process affected a considerable length of the media in the section taken and about one-fourth of its thickness. In the necrotic media at the base of the thrombus were many pyknotic nuclei, mostly of polymorphonuclear cells. A similar process affected most of the outer layer of the media at the level of the dissection. This necrotic process was not confined to any one segment of the aorta but was seen at several levels below the dissection. In an elastic-connective tissue stain, the elastic fibers were almost undamaged, but the intercellular substance appeared to have suffered the chief alteration. In the adventitia was a sparse round cell infiltration no more than that normally present.

Comment.—The areas of early necrosis in the aorta probably resulted from an impaired nutrition of the wall secondary to the dissection. This was suggested by their occur-

rence beneath the adherent thrombus and not to the same degree elsewhere.

Diagnoses.—1. Dissecting aneurysm of the aorta with rupture into the left pleural sac. 2. Hemothorax, left. 3. Ecchymosis into the posterior mediastinal, retroperitoneal, and pericardial tissues. 4. Arteriosclerosis of the aorta, mild. 5. Infarct, right kidney.

DISCUSSION

These two reported cases, and in addition one other case of dissecting aortic aneurysm discovered at post-mortem examination in 1936, constitute the total number of such cases observed in 31,300 admissions over a period of ten years. During this time there were 1421 deaths and 360 necropsies, giving an incidence of 0.84 per cent of the autopsied material.

The third case referred to was found in a 38-year-old white male, who had a moderate hypertension and died suddenly. The details of his clinical course are not available. Post-mortem examination revealed a tense cardiac tamponade, due to a hemopericardium. The aorta showed two transverse tears 1 cm. above the aortic ring, the edges of which were smooth, as though cut by a knife. The adventitia was separated from the media, forming a second coat which extended from the intimal tears to the diaphragmatic level, encircling the aorta for approximately three-fourths of its circumference. In the descending aorta, 15 cm. from the arch, there were three more transverse intimal tears, which were, also, smooth. There was no evidence of syphilis. Microscopic examination revealed endothelialization of the new tube, a priori evidence that the condition was not very recent. No atherosclerosis of the new aorta was present, as it was in the case recently reported by Graybiel and Sprague.[6] The heart weighed 350 grams. The coronary arteries showed advanced atherosclerotic changes.

In these three cases, hypertension of severe degree was present in two, and of moderate degree in one. The marked variation in the degree of atherosclerosis was striking, being most severe in the case illustrated, and practically absent in the second case. In the third case it was present in moderate degree.

Another interesting point was the fact that the intimal tears in all three cases occurred in a portion of the aorta which was relatively free from atherosclerotic changes. The explanation for this is obscure. The margins of these rents were straight and sharp, all conforming to the type depicted in the illustration. None of the vessels showed any of the acceptable criteria for a diagnosis of syphilitic aortitis, the serology being negative in all three, although a history of inadequately treated lues was obtained from one (Case 1). All three showed some degree of perivascular round cell infiltration in the adventitia, mild in two and moderate in the third (Case 1). There was no dilatation of the aortic ring, however, and there was no separation of the valve commissures.

While, as stated before, the presence of a syphilitic mesaortitis may enhance dissection, Klotz and Simpson[7] state that the luetic process, being a "granulomatous inflammatory process tends to weld the lamellae more closely together,"[8] so that "the wall would split less readily into its anatomic layers."

It is to be noted that the signs which may accompany this syndrome depend, in part, upon the site of the intimal tear, which usually conforms to the level of the origin of the dissection. Its subsequent course is of marked importance and varies to a considerable degree in different cases. These facts are brought out vividly when the two reported cases are compared. In Case 1, the

intimal tear occurred slightly above the aortic ring, the more usual site, resulting in the sudden appearance of a systolic and diastolic murmur. With the progression of the dissection, the likelihood of involvement of the larger vessels of the upper extremity is good, explaining the marked disparity in blood pressure readings obtained in both arms. While the blood pressure was not taken in both arms in the second case, it is doubtful, from the necropsy findings, whether any disparity had existed. Obviously, if the iliacs are involved in the dissection, differences in the pulsations of the arteries of the lower extremities may be apparent. This may or may not accompany a sensation of numbness, tingling, or coldness of the involved extremity, as was present in the first case. It was not looked for in the second case.

The striking findings in these cases were a history of severe thoracic pain, with widespread, variable radiation, the absence of electrocardiographic evidence suggesting a "protracted coronary insufficiency," and the continuation of an unusually marked, pre-existing hypertension. Both patients were obviously ill even after the pain had subsided. In the first case, the actual dissection and rupture were observed while the patient was on the ward. The additional findings of the newly developed murmurs, the disparity in blood pressure readings, and the paraesthesias and coldness of the left leg made the diagnosis relatively obvious.

SUMMARY

1. Two cases of massive dissection of the aorta are presented, in one of which an ante-mortem diagnosis was made. The pathologic description of a third case is given.

2. An idea of the incidence of this entity, and the difficulties in diagnosis are brought out. Certain factors suggesting the diagnosis are discussed.

I am indebted to Dr. Roy Barnett for the pathologic description of the heart and aorta.

REFERENCES

1. Flaxman, N.: Dissecting Aneurysm of Aorta, Am. Heart J. 24: 654, 1942.
2. Shennan, T.: Dissecting Aneurysm, London, 1934, Medical Research Council.
3. Weiss, S.: Dissecting Aneurysm of the Aorta; Two Cases with Unusual Features, New England J. Med. 218: 512, 1938.
4. Boyd, Wm.: Textbook of Pathology, Philadelphia 1934, Lea & Febiger.
5. Pardee, Harold E. B.: Clinical Aspects of the Electrocardiogram, New York, 1941, Paul B. Hoeber.
6. Graybiel, Ashton, and Sprague, Howard B.: Dissecting Aneurysm of the Aorta, Am. Heart J. 21: 530, 1941.
7. Klotz, O., and Simpson, W.: Spontaneous Rupture of the Aorta, Am. J. M. Sc. 184: 455, 1932.
8. Niehaus, Frederick W., and Wright, Willie D.: Dissecting Aneurysm of the Aorta, J. Lab. & Clin. Med. 26: 1248, 1941.
9. Pekin, Thomas J., and Nesbitt, John W.: Dissecting Aneurysm of the Aorta, Md. Bull. Vet. Admin. 19: 96, 1942.
10. Reiss, Jack: Aneurysm of the Aorta; Two Cases, M. Bull. Vet. Admin. 18: 272, 1942.

THE EXPLANATION FOR THE ABSENCE OF CLOTTING IN BLOODY CEREBROSPINAL FLUID*

M. J. MADONICK, M.D.,† AND BERNARD NEWMAN, CH.E., M.S.‡
NEW YORK, N. Y.

ALTHOUGH textbooks of neurology state that the spinal fluid in cases of subarachnoid bleeding does not clot on standing and that spinal fluid admixed with blood which is produced by trauma during lumbar puncture does clot on standing, very little is reported in the literature regarding the explanation for the lack of clotting in the former.

Fresh bloody cerebrospinal fluids of fifteen patients, varying in age from 20 to 60, were studied. No clotting occurred after the lapse of many hours in any of the specimens. Slight xanthochromia was noted in the supernatant fluid of one of the specimens. Examination of the smeared sediment with Wright's stain revealed the presence of many erythrocytes, normal in size, shape, color, and staining properties, a moderate distribution of leucocytes, and what appeared to be a dearth of thrombocytes.

EXPERIMENTAL PROCEDURE

In five of the fluids selected at random, the calcium concentration varied from 4.3 to 5.1 mg. per 100 c.c. In two of the fluids, the cholesterol was either zero or a slight unreadable trace.

The cerebrospinal fluids were examined both before and after centrifuging for the presence of fibrinogen by adding to each 0.5 c.c. of fluid varying amounts of M/40 $CaCl_2$, from 0.1 to 0.5 c.c., and 0.2 c.c. of thromboplastin suspension made up according to Ziffren et al.[14] In no case did clotting of the cerebrospinal fluid occur.

It was also considered possible that an anticoagulant was present in the spinal fluid which may have accounted for the failure to clot spontaneously. Accordingly, 0.5 c.c. of the fluid was added to 2 c.c. of freshly drawn blood and permitted to stand at room temperature. In all eight of the cases in which this was done, clotting took place. The presence of such a heparin-like anticoagulating factor in the cerebrospinal fluid after nontraumatic subarachnoid hemorrhage may thus be ruled out.

In order to determine whether the amount of blood in the hemorrhagic spinal fluid would not give enough fibrinogen to permit clotting, nontraumatic spinal fluids from three normal individuals in the same age group were colored with freshly drawn blood to about the same intensity as the hemorrhagic fluids

*From the Neuropsychiatric Service of the Morrisania City Hospital, Nathan Savitsky, M.D., Chief, and the Research Laboratory of the Hospital of the Daughters of Jacob, New York City.

†Assistant Visiting Neuropsychiatrist, Morrisania City Hospital, Instructor in Neurology, College of Physicians and Surgeons, Columbia University.

‡Chemist at Hospital of Daughters of Jacob.

Received for publication, June 8, 1943.

and permitted to stand. In all three of these cases, the fluids clotted in about twenty minutes to a jelly-like consistency.

In the next series of studies performed, blood plasma was added in the proportion of 0.5 c.c. to each 2 c.c. of clear centrifuged hemorrhagic spinal fluid to determine whether the criterion for clotting was the presence or absence of fibrinogen. When this was done and 0.2 c.c. of M/40 $CaCl_2$ added, the fluids clotted, but only on addition of a thromboplastin suspension. However, if to the uncentrifuged spinal fluid only plasma and $CaCl_2$ were added, omitting the thromboplastin, clotting also took place. In other words, except for fibrinogen, everything necessary for the clotting of the spinal fluid was present in the fresh hemorrhagic fluid.

The calcium determinations obtained in the fluids tested were within normal limits (Merritt and Bauer[7]). There were no alterations in the cholesterol content (Plaut and Rudy[10]). Lamb[4] emphasized the absence of clot formation in the spinal fluid of meningeal hemorrhage. He performed several experiments with fluids and blood, stating that "even though the amount of blood obtained in spontaneous meningeal hemorrhage be much greater than that required to clot a mixture of normal blood and fluid in vitro, no clotting takes place." He advanced no explanation for this phenomenon.

Our experiments indicate that the lack of clotting is due to the absence of fibrinogen. Normal spinal fluid does not contain this substance (Levinson,[5] Merritt and Fremont-Smith,[8] Strauss and Kaliski[13]). We then have to explain what happens to the fibrinogen of the blood that escapes from the source of the bleeding. We believe that there is clot formation in the cranial cavity and that the fibrinogen is thus removed. The blood that is obtained on lumbar puncture is therefore serum with red blood cells. On autopsy, performed on one of the two fatal cases in our series, clots were found in the subarachnoid spaces. Clots and hematomas in the subarachnoid spaces and even in the ventricles have been frequently described in cases of subarachnoid hemorrhage (Bagley,[1] Fearnsides,[2] Schmidt,[11] Strauss et al.[12]). The explanation for the lack of clotting is the same as that suggested by Neel[9] and by Froin.[3] The former stated that a nonartificial admixture of blood and cerebrospinal fluid "does not coagulate because coagulation has already taken place in the subarachnoid space." However, he gives no data to show how he reached this conclusion. Froin offered the explanation that in subarachnoid hemorrhage the blood forms a clot at and around the site of leaking. He further stated that the proportion of red cells comprising the clot varies according to the severity and the site of the hemorrhage. If the latter be in one of the basal cisterns where the circulation of cerebrospinal fluid is relatively unimpeded, large numbers of erythrocytes are washed off and rapidly diffuse through the subarachnoid space. If on the other hand the site of the bleeding be in the lateral ventricle, the channel of flow is relatively narrow and the great majority of red cells remain trapped in the clot. The red cells that reach the basal cistern then rapidly diffuse through the subarachnoid spaces.

We believe the mechanism is similar to the absence of clotting in menstrual blood (Lozner et al.[6]). Thromboplastin has to be added to the menstrual blood, but its addition does not seem to be necessary for clot formation in the cerebrospinal fluid.

CONCLUSION

Fresh bloody spinal fluids of fifteen patients with meningeal bleeding were examined to determine the cause of the lack of clotting. It was found that fibrinogen was absent in all cases. This, we believe, is due to the occurrence of clotting in and around the brain during which process the fibrinogen is removed from the fluid.

REFERENCES

1. Bagley, C.: Spontaneous Cerebral Hemorrhage, Arch. Neurol. & Psychiat. 27: 1133, 1927.
2. Fearnsides, E. G.: Intracranial Aneurysms, Brain 39: 224, 1916.
3. Froin, G.: Les Hemorrhagies Sous-Arachnoïdiennes et Le Mécanisme de l'Hematolyse en général, Faculté de Medecin de Paris, Thèse 1904.
4. Lamb, F. H.: Spontaneous Meningeal Hemorrhage, J. Lab. & Clin. Med. 15: 531, 1930.
5. Levinson, A.: Cerebrospinal Fluid in Health and in Disease, St. Louis, 1929, C. V. Mosby Co., pp. 61, 89.
6. Lozner, E. L., Taylor, Z. E., and Taylor, F. H. L.: The So-called "Coagulation Defect" in Menstrual Blood, New England J. Med. 226: 481, 1942.
7. Merritt, H. H., and Bauer, W.: The Equilibrium Between Cerebrospinal Fluid and Blood Plasma. III. The Distribution of Calcium and Phosphorus Between Cerebrospinal fluid and Blood Serum, J. Biol. Chem. 90: 215, 1931.
8. Merritt, H. H., and Fremont-Smith, F.: The Cerebrospinal Fluid, Philadelphia, 1938, W. B. Saunders Co., p. 30.
9. Neel, A. V.: The Content of Cells and Proteins in the Normal Cerebrospinal Fluid, London, 1939, Oxford University Press, p. 118.
10. Plaut, F., and Rudy, H.: Über die Beziehungen zwischen Blutcholesterin und Liquorcholesterin, Ztschr. f. d. ges. Neurol. u. Psychiat. 146: 262, 1933.
11. Schmidt, M.: Intracranial Aneurysms, Brain 58: 489, 1930-31.
12. Strauss, I., Globus, J. H., and Ginsburg, S. W.: Spontaneous Subarachnoid Hemorrhage, Arch. Neurol. & Psychiat. 27: 1080, 1927.
13. Strauss, I., and Kaliski, D. J.: The Reaction of the Human Cerebrospinal Fluid in Infectious Inflammatory Conditions of the Meninges, A. Research Nerv. & Ment. Dis. 4: 269, 1924.
14. Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P.: Control of Vitamin K Therapy. Compensatory Mechanisms at Low Prothrombin Levels, Proc. Soc. Exper. Biol. & Med. 40: 595, 1939.

THROMBOPHLEBITIS MIGRANS*

Morgan Yale Swirsky, M.D., and Caesar Cassano, M.D., New York, N. Y.

THROMBOPHLEBITIS migrans is a relatively uncommon condition of unknown etiology. It involves chiefly the superficial veins of the upper and lower extremities. Widely separated regions of the body are affected with segments of normal veins between the regions of thrombosis. There is apparently no relation to pre-existing disease of or trauma to the vessels. The veins of the visceral organs are very rarely involved. Tendency to embolize is stated by many observers to be infrequent, while others stress the frequency with which it occurs in the lungs. The disease affects both sexes and all ages. The course is of variable duration and is generally benign, usually terminating in complete recovery. Approximately 100 cases have been reported in medical literature, but in only four acceptable instances has autopsy been performed.[1-4] We *disagree* with Birnberg and Hansen in accepting the case of Hirschhorn et al., because the rheumatic lesion was completely healed, was not more severe than has frequently been found in other autopsies as an incidental observation, and the acute lesion of the heart was nonrheumatic in nature. The present communication is the report of another case of thrombophlebitis migrans with autopsy. The rarity of necropsy observation seems to justify its presentation.

CASE REPORT

The patient, A. C., a 27-year-old white female, was admitted to the medical service of Dr. John Carroll, on March 7, 1942, with a chief complaint of pain and swelling of the right leg and thigh. This was of 1½ weeks' duration. For two weeks prior to the onset, she had been confined to bed with urinary frequency, urgency, burning, and nocturia. Pain which was first noted on the inner aspect of the right ankle joint soon spread up the entire lower limb as high as the groin and was accompanied by swelling of the extremity; chills, fever, and diaphoresis were absent. There was no history of trauma or any previous similar episode.

Past History.—At the age of 6 to 7 years, she had an attack of chorea without polyarthritis or apparent rheumatic heart disease. At the age of 17, shortly after the death of her mother, the patient began to gain weight and soon became very obese. Since the age of 20, she had suffered with attacks of bronchial asthma.

Physical Examination on entry revealed a very obese young white female, appearing somewhat breathless, with dark rings around her eyes. Pupils were round, regular, equal in size, and reacted well to light and accommodation. Oral hygiene was poor and the pharynx was somewhat injected. The neck was supple and no nodes were palpable. The heart was apparently not enlarged, rhythm was regular, tones were of good quality with no audible murmurs, and P_2 was greater than A_2. Blood pressure was 180 systolic and 120 diastolic. Both lung fields were full of asthmatoid wheezes and harsh breath sounds. Numerous white striae were present over the abdomen; palpation was negative. The right lower extremity showed generalized edema and local heat with exquisite tenderness to touch along the course of the saphenous vein. The left lower extremity was not remarkable. There was

*From the First Medical Division and the Pathological Laboratory, City Hospital, Welfare Island, Dept. of Hospitals.

Received for publication, June 11, 1943.

a moderate degree of clubbing of the fingers. No abnormal hirsutism was noted. Temperature was 99.3, pulse, 128, with respirations at 20.

Clinical Course.—Patient was put to bed and treated with palliative measures for three days. The fever rose slowly but steadily during this period to 103.0 accompanied by a rapid pulse rate out of proportion to the temperature, and with increasing dyspnea and cyanosis. The physical findings were suggestive of hypertensive heart disease with left ventricular failure, so-called "cardiac asthma," and a right lower lobe pneumonitis. She was treated with the usual measures with no startling results. A roentgenogram of the chest taken on the third hospital day showed diffuse irregular confluent densities throughout the right lung field, enlargement of the heart with left ventricular predominance, and hepatomegaly. The electrocardiogram revealed a simple tachycardia with a rate of 136, A-V conduction time of 0.16 seconds, slight left axis deviation, diphasic T_1, and inverted Q_3 and T_3. The blood Wassermann reaction was negative, nonprotein nitrogen, 27; sugar, 114; cholesterol, 85; cholesterol esters, 29; icteric index, 15; calcium, 9.7; phosphorus 2.56; albumin, 3.2 per cent; and globulin, 1.9 per cent. The urinalysis revealed a specific gravity of 1.022, two plus albumin, and numerous pus cells. A sputum typing was negative for pneumococci, but showed a *Staphylococcus aureus*. Despite the use of oxygen, digitalis, chemotherapy, and various supportive measures, the patient remained quite uncomfortable and ran an irregular febrile course with accompanying comparable pulse rate. The temperature ranged between 99.2 and 102.6 degrees and the pulse rate between 98 and 130 per minute. A second roentgenogram of the chest taken on the sixth hospital day showed slight clearing of the bronchopneumonic changes.

On the fourteenth hospital day, she suddenly complained of sharp and rather severe pain in the right lower chest. Examination revealed dullness to percussion with scattered moist râles over the right lower lobe, a tender liver enlarged three fingerbreadths below the costal margin, and bilateral ankle edema, more marked on the right side. The chest roentgenogram showed a consolidating process in the right lung field, while the electrocardiogram revealed depressed T_1 and T_2 but otherwise was similar to the previous tracing. Blood and urine cultures were negative although the urine continued to show numerous pus cells, albumin, and a specific gravity of 1.016. The icteric index was 18, nonprotein nitrogen, 35; cholesterol, 143; and cholesterol esters, 40. Red blood cells were 4,400,000; hemoglobin, 70 per cent (Sahli); and white blood cells, 15,000, with 83 per cent polymorphonuclears. Sedimentation time was normal. On the eighteenth day, she complained of sudden onset of severe pain in the posterior aspect of the left thigh and calf with palpatory tenderness along the course of the saphenous vein. During the following twenty-four hours, the usual findings of a thrombophlebitis appeared. She received a course of heparin ("Liquaemin") with good symptomatic relief. The irregular low-grade febrile course and accelerated pulse continued after subsidence of the thrombophlebitis, and episodes of marked dyspnea and some cyanosis continued, accompanied by scattered râles and wheezes in both lung fields and persistently elevated blood pressure of 200/126 to 250/120. The cardiac physical findings were not noteworthy.

On the forty-first day, severe pain in her left upper extremity suddenly occurred. The arm was greatly swollen, hot to touch, exquisitely tender to light palpation, and had a peculiar mottled cyanosis of the skin overlying the involved area. Another course of heparin ("Liquaemin") was given, again with dramatic symptomatic relief, and this thrombophlebitic incident apparently rapidly subsided. On the fifty-first day, she developed signs of a left upper lobe pulmonary infarction which caused her transient discomfort. On the fifty-seventh day, thrombophlebitis of the left external jugular vein developed suddenly, accompanied by severe pain and reappearance of the swelling of the left arm. A blood count the following day showed red blood cells of 3,450,000 and leucocytes of 14,300 with 85 per cent polymorphonuclears. *Staphylococcus aureus* and *Bacillus coli* were isolated from the blood. She gradually lapsed into coma, temperature and pulse rate rose steadily, and the patient expired on the sixty-first day in pulmonary edema. Final temperature was 105, pulse, 142, and respirations were 50.

Autopsy.—(Performed eighteen hours after death. Only the pertinent data are abstracted.) The body is that of a markedly obese white woman of about the stated age. There

is a two-plus pitting edema of the left arm and both legs. The right pupil is slightly larger than the left. The peritoneal cavity contains one liter of clear straw-colored fluid.

Heart.—750 grams. The pericardial sac contains 125 c.c. of a clear straw-colored fluid. The heart is enlarged in all chambers. The right auricle is covered with a thin layer of fibrin. There is abundant epicardial fat. On the anterolateral wall of the right auricle is a firmly attached friable gray thrombus covering the endocardium. The remainder of the mural endocardium is normal. The mitral valve is thickened, and along the line of closure is a row of pinhead-sized firm vegetations similar in color to the body of the cusps. On the auricular surface of the posterior cusp above the line of closure is a single larger vegetation, 1 cm. in length, firmly attached, gray-pink, and somewhat friable. The chordae tendineae are normal. The aortic, tricuspid, and pulmonic valves are normal. The left ventricle is markedly and the right ventricle moderately hypertrophied. The myocardium is of good tone and color and appears free of scar. The coronary arteries have mild to marked patches of atheromatous thickening.

Aorta.—There is an occasional atheromatous plaque in the arch and abdominal portion. The main branches are slightly dilated.

Veins.—The left jugular and innominate veins down to the junction with the superior vena cava contain firmly attached thrombi with reddish cores. Firm gray thrombi occlude the right subclavian and both femoral veins. The venae cavae, portal and hepatic veins are normal.

Lungs.—Right 975 Gm., left 550 Gm. Each pleural sac contains one liter of clear straw-colored fluid and has readily torn stringy fibrous adhesions. The posterior surface of the right lower lobe is covered with fibrin. In the right lung are many areas, irregular in size and shape, firm, granular, and friable; some are gray in color. Near the hilum is a roughly pyramidal area of the lower lobe with the base resting on the horizontal fissure. The center is gray-pink; the periphery, hemorrhagic. In both lungs, many emboli are present in the medium and smaller branches of the arteries. The tracheobronchial tree has copious quantities of seromucus, and the mucosa is mildly congested. The lymph nodes are enlarged, edematous, and blue-black.

Liver.—2,000 Gm. There is an intensification of the markings, giving it a typical nutmeg appearance. The extrahepatic biliary system is negative.

Spleen.—375 Gm. The capsule is tense, irregularly thickened, and has a few stringy fibrous adhesions to the diaphragm. The cut surface has a dark mahogany color and soft consistency. In the subcapsular zone are two small fresh infarcts, one with a gray, the other with a hemorrhagic center. The artery and vein are free of thrombosis.

Urinary System.—Right kidney, 215 Gm.; left kidney, 240 Gm. The fibrous capsules are slightly adherent, leaving a moderately coarse granular surface. The cut surfaces are edematous, but otherwise normal. The lower third of the left ureter is slightly dilated. Otherwise, the system is natural.

G. I. Tract.—There is diffuse submucosal congestion.

Brain.—1,250 Gm. The meninges are markedly engorged. Section fails to reveal any other changes. The pituitary is normal.

Paranasal Sinuses.—The sphenoidal sinus contains a light brown, moderately thick, purulent exudate which flows freely upon opening the sinus. The ethmoids, middle ears, and mastoids are normal.

Pancreas, adrenals, and generative organs are normal.

Histology.—Heart.—The left ventricle has marked hypertrophy; the right, moderate. Small perivascular and interstitial scars are present, most abundant in the interventricular wall and left papillary muscles. In a few there are small foci of lymphocytes, occasionally with mast cells. There are a few isolated foci of polymorphonuclears in the subendocardial zone of the right ventricle. Some of the intrinsic arteries have slight endothelial hyperplasia. The right coronary artery has marked atheroma and dense lymphoid foci in the adventitia. The mitral valve is thickened, fibrotic, and hyaline. It is well vascularized with thin-walled vessels and small thick-walled arteries, having a fine intimal elastification. The small vegetations are dense and fibrotic. The larger vegetation is similar and covered with a platelet thrombus.

Lungs.—There is diffuse capillary engorgement. In the right lung are foci of hemorrhage completely destroying the architectural pattern, surrounded by alveoli containing edema fluid, polymorphonuclears, and hemosiderin-bearing phagocytes. Many of the smaller arteries are occluded by well-organized, partially recanalized thrombi. It both lungs are foci of consolidation surrounding smaller bronchi.

Liver.—There is marked diffuse chronic passive congestion. The cords of the central zone show dissociation, necrobiosis, and bile pigmentation. The portal areas are not remarkable.

Spleen.—There are several small acute infarcts. The remaining portions show moderate interstitial fibrosis, engorged sinusoids, reduction of follicles, and normal central arteries.

Kidneys.—A few of the afferent arterioles have moderately thickened media.

Sphenoid Sinuses.—There is extreme destruction of the architecture of the mucosa with heavy polymorphonuclear, lymphocytic, and plasmacytic infiltration.

Left Internal Jugular Vein.—The lumen is occluded by a thrombus whose peripheral portion is organized and recanalized and has small foci of lymphocytes. The central portion has well-preserved and ghost-like red blood cells. The wall of the vein is edematous and has a mild lymphocytic infiltration.

The other organs show only extreme engorgement.

Anatomical Diagnosis.—Thrombophlebitis migrans; cardiac hypertrophy; healed rheumatic heart disease; recent and old pulmonary emboli; acute bronchopneumonia; generalized chronic passive engorgement; splenomegaly; acute infarcts of spleen; chronic sphenoiditis; obesity.

COMMENT

This case presented many of the well-recognized features of migrating thrombophlebitis and several less common findings. As has been noted frequently, the acute episodes were recurrent in nature and associated with fever. The veins involved were in widely separated regions of the body and not entirely confined to the more superficial ones. As usual, the process appeared first in the subcutaneous veins of one leg and then, by a series of relapses, involved the superficial veins of all the other extremities. Thrombosis of the deep veins, i.e., the femorals, subclavian, innominate, and left external jugular veins, occurred, associated with edema, but without gangrene. No evidence of phlebitis or thrombosis of the visceral[5, 6] or coronary veins[7-10] was noted, although the possibility of these types of involvement has been repeatedly suggested by several authors. Multiple pulmonary and splenic infarcts were present, but no evidence of thrombophlebitis or thrombosis of the pulmonary veins[11, 12] could be detected, so that the only plausible etiology must be embolism from peripheral thrombi. The clinical cardiac findings in this case coupled with those at necropsy had no fundamental relationship to the migrating thrombophlebitis.

The etiology of this disease still remains unsolved, although numerous exhaustive studies have been done on the possibilities of disturbance of clotting mechanism, focal infection, allergic types of sensitivity reaction, and fundamental relationship to Buerger's disease as primary causative factors. Many investigators have taken repeated cultures during the acute phases in the hope of proving a bacteremia or septicemia as an etiologic agent, but the results have been very disappointing. Hedblom[13] reported the presence of "nonmotile spore formers" in the blood stream in his study, and Walker,[14] the finding of *Bacillus alkaligenes*. In our case a blood culture taken early in the course of the disease showed no growth, while a culture taken forty-eight hours before death

showed *Staphylococcus aureus* and *Bacillus coli.* This may have been an agonal phenomenon, and no definite conclusions can be drawn. Unfortunately, cultures from the involved veins were not taken at the autopsy table. The rheumatic lesions found at autopsy appeared to be purely incidental findings having no relationship to the thrombophlebitis. Our conclusions were similar to those adopted in reference to the case of Hirschhorn et al.

The treatment in these cases still leaves much to be desired. Our patient received adequate heparinization, but this treatment failed to prevent further recurrences and thromboses. However, dramatic and rapid symptomatic relief was definitely obtained from the use of this agent.

REFERENCES

1. Lipschitz, M.: Zur der Thrombophlebitis Migrans, Deutsche med. Wchnschr. 55: 744, 1929.
2. Stern, N. S.: Thrombophlebitis Migrans, With Report of Two Fatal Cases With Autopsies; One Also Showing Primary Carcinoma of the Liver, South. M. J. 27: 849, 1934.
3. Hirschhorn, L., Lisa, J. R., and Goldstein, N. J.: Thrombophlebitis Migrans; Case Report, Am. Heart J. 17: 76, 1939.
4. Birnberg, V. J., and Hansen, A. E.: Thrombophlebitis Migrans, J. Pediat. 21: 775, 1942.
5. Kletz, N.: Thrombo-phlebitis Migrans, The Med. Press and Circular 192: 371, 1936.
6. Kletz, N.: Thrombo-phlebitis Migrans, Lancet 2: 938, 1932.
7. Moorehead, T. G., and Abrahamson, L.: Thrombophlebitis Migrans, Brit. M. J. 1: 586, 1928.
8. Hartfall, S. J., and Armitage, G.: Thrombo-phlebitis Migrans; Report of 2 Cases, Guy's Hosp. Rep. 82: 424, 1932.
9. Ryle, J. A.: Thrombophlebitis Migrans, Lancet 2: 731, 1930.
10. Campbell, M., and Morgan, O. G.: Unusual Case of Multiple Thrombosis, Guy's Hosp. Rep. 80: 34, 1930.
11. Collier, W. T.: Thrombophlebitis Migrans, Involving Deep Veins of All Four Limbs, Lancet 2: 1408, 1931.
12. Ellison, J. B.: Thrombo-phlebitis Migrans Complicating Scarlet Fever, Brit. J. Child. Dis. 28: 207, 1931.
13. Hedblom, C. A.: A Case of Thrombophlebitis Migrans, J. A. M. A. 66: 1777, 1916.
14. Walker, A. B.: Observations on Thrombophlebitis Migrans With Notes of Case, Lancet 2: 936, 1932.

THE ARTIFICIAL PHOSPHOLIPID MEMBRANE, SEMIPERMEABILITY, AND THE BLOOD-BRAIN BARRIER*

J. H. WEATHERBY, PH.D., RICHMOND, VA.

INTRODUCTION

IN A previous paper by the author (1943) it was pointed out that collodion membranes containing lecithin and cephalin exhibit some of the properties of semipermeability generally associated with membranes of living cells. Among those properties found to be common to both artificial and natural membranes may be mentioned the permeability characteristics with respect to weak acids and weak bases and the behavior of the asymmetry potential under various conditions of pH. Weak acids, such as salicylic, permeate the artificial membrane only when pH of the solution permits their existence as such and not as salts; that is to say, permeation occurs from an acid medium in which much of the acid exists as un-ionized molecules, and not from an alkaline medium in which the degree of ionization is very high. Similarly, weak bases, such as nicotine, permeate the artificial membrane only when pH of the solution permits their existence in the molecular form and not as ions; that is, permeation occurs from an alkaline medium and not from an acid one. The explanation proposed for these phenomena is based on ionization of the lecithin or cephalin molecules contained in the membrane, the nature of the ionization depending on pH of the medium in contact with the membrane. This is believed to give rise to the asymmetry potentials observed in membranes, both artificial and natural. Ions bearing charges of similar sign to that of the membrane are repelled by the membrane, and in this manner penetration of the membrane is prevented. Un-ionized molecules are not repelled in this manner, however, and penetration may occur more or less readily. A definite correlation has been found to exist between rate of penetration and concentration of un-ionized molecules of acid or base.

If the above is true, then by making relatively simple changes in molecules it should be possible to change the permeating characteristics of the molecules. For example, derivatives of nicotine in which methyl iodide is added to one or the other, or both the nitrogen atoms contained in the molecule, would be expected to ionize under all conditions of pH which do not actually destroy the molecule, and consequently should not permeate membranes containing lecithin and cephalin under such conditions. Also, the esterification of salicylic acid as in the production of methyl salicylate, or the removal of the carboxyl group to form phenol, should result in permeation of membranes by these substances under all conditions of pH which do not destroy the molecules. Some of the experiments to be described herein are offered as evidence supporting these conclusions.

*From the Department of Pharmacology, Medical College of Virginia.
Received for publication, June 22, 1943.

In a recent review Friedemann (1942) pointed out that many substances, when introduced into the blood stream, pass from blood into brain substance, while certain others do not. The many observations by various authors referred to in this review have resulted in the postulation of the existence of a blood-brain barrier which is highly selective in its ability to permit or deny passage of substances into the brain from the blood. It is interesting to note that among substances which pass from blood to brain are the alkaloids, basic dyes, and certain toxins. Among others which do not readily permeate the brain are the acid dyes, the arsphenamines, a few toxins, and most or all the viruses. The examination of the formulas and physicochemical properties of these various substances, as far as they are known, shows that those which penetrate the brain exist at body pH, at least in part, as un-ionized molecules; whereas, those which do not penetrate exist largely in the ionic forms. Friedemann and Elkeles (1934) found that the ability of toxins to penetrate the brain is closely related to their electrical status. Those which are negatively charged do not penetrate the brain; whereas, those which are positively charged or electrically neutral at body pH do penetrate. Presumably these electrical charges may be due either to ionization of one group or another of the toxin molecule, or to adsorption of ions. An electrical charge would result in either event. The nonpermeation of viruses, as pointed out by Friedemann, may be due to the relatively large sizes of the particles; but it is interesting to note that of those for which such information is available, all are negatively charged. Certain others of the nonpermeating substances, such as trypan blue, congo red, and probably the arsphenamines, are believed to be of colloidal size, and hence do not permeate normal membranes. These facts suggest that the blood-brain barrier may be nothing more nor less than cell membranes containing phospholipids. Obviously, then, any cell membrane containing such substances would be expected to serve as such a barrier. Some of the experiments to be described are believed to have a direct bearing on this question.

EXPERIMENTAL METHODS AND RESULTS

Collodion membranes containing lecithin and cephalin (from brain) were prepared according to methods previously described (Weatherby, 1943) and were used in connection with dialysis cells such as those used previously. An effort was made to include sphingomyelin and cholesterol in some of these membranes, but was abandoned because sphingomyelin is not appreciably soluble in the ether-alcohol solvent used for collodion, and cholesterol crystallizes when the ether content of the solvent is somewhat reduced by evaporation.

Three methyl iodide derivatives of nicotine were tested, as well as nornicotine. Permeability of collodion membranes containing lecithin and cephalin to these nicotine derivatives, as well as various other substances reported either to penetrate the brain or not to penetrate it when injected intravenously, was ascertained by placing solutions of these substances on one side of the membranes and distilled water on the other. The dialysates were examined at intervals for the various substances. Generally two solutions of each substance were used, one at approximately body pH and another that was definitely acid, that is, pH 3.5 to 4.5. The results of these experiments are shown in Table 1.

An examination of this table shows that those substances which exist as salts at body pH, and are therefore subject to ionization, do not readily pass through the artificial membrane; whereas, those which exist partly or largely as un-ionized molecules at this pH do penetrate these membranes. The parallelism between penetration or nonpenetration of many of these substances into the brain from the blood stream and the permeation or nonpermeation of these same substances with respect to the artificial membrane is striking indeed. Since most, if not all, cell membranes are believed to contain phospholipids (with some, their presence has been demonstrated conclusively), it seems probable that the blood-brain barrier is one or more membranous systems, which owe their selective properties in a large measure to their phospholipid contents.

TABLE I

SUBSTANCE	pH 3.5-4.5	pH 7.3-7.4	PASSAGE OF BLOOD-BRAIN BARRIER (FRIEDEMANN, 1937)
Nicotine methyl iodide derivatives*	0	0	
Nicotine	0	+ +	
Nornicotine*	0	+	
Phenol	+ +	+ +	
Neoarsphenamine	precipitated	0	
Mapharsen	+	+ +	
Methylene blue	+	+ +	+
Toluidine blue	trace	+ +	
Methyl red†	trace	+ +	
Neutral red	0	+ +	+
Eosin	+ +	+	−
Trypan blue	0	0	−
Congo red	0	0	−

All concentrations 0.25 per cent, except as indicated.
*Molecular equivalent of 0.25 per cent nicotine.
†Saturated.

DISCUSSION

Several of the experiments reported in the table deserve discussion in greater detail. Trypan blue and congo red are colloidal, and hence could not be expected to permeate any but the most highly permeable membranes, although it is well known that the former readily passes through the damaged capillaries in an inflamed area. One would expect that neoarsphenamine, which exists as a salt in an alkaline medium, would be changed into the less highly ionizable acid form in an acid medium and that permeation of the artificial membrane would occur under these conditions. However, precipitation of neoarsphenamine (probably following decomposition) occurred within a few hours after acidification of the solution, and no permeation occurred within twenty-four hours. Methyl salicylate would be the substance to be preferred instead of phenol for comparison with salicylic acid, but this ester is not sufficiently soluble in water for such an experiment. None of the three methyl iodide derivatives of nicotine were found to permeate the artificial membrane.

With nicotine and nornicotine, just as with all other organic bases, the ratio between free base and salt at any given pH may be obtained by means of the Henderson-Hasselbalch equation, $pH = pK + \log \frac{\text{Base}}{\text{Salt}}$. The reciprocal

of this relationship holds for weak acids. The value of pK for nicotine is approximately 8.07; that is, half is free base and half salt at this pH. With nornicotine the value of pK is 9.09. At pH 7.4 approximately 18 per cent of the nicotine exists as free base, while at the same pH only about 2 per cent of nornicotine exists as such. Nornicotine permeated the artificial membrane at body pH, but much more slowly than nicotine under similar conditions. However, when pH of the nornicotine solution was increased to 9.5, the rate of permeation was greatly increased, and under these conditions it compared favorably with the rate of permeation of nicotine from a solution containing the same proportion of free base. In this connection it is interesting to note that the L.D. 50 for nicotine on intravenous injection into rabbits is approximately 6 mg. per kg., while for nornicotine it is about 3 mg. However, on intraperitoneal injection, doses of 15 mg. per kg. of nicotine killed six of ten rabbits; whereas, nornicotine in molecular equivalent doses (13.7 mg. per kg.) killed only one of ten.* Thus, nornicotine is about twice as toxic as nicotine on intravenous injection; but when passage through additional membranes is necessary before its toxic properties can be exhibited, as on intraperitoneal injection, nornicotine is much less toxic than nicotine.

It has been well established by many experiments reported by various authors that the rates of absorption of other alkaloids are dependent on pH of the media containing the alkaloids. This suggests the interesting possibility that pharmacologic activities of many alkaloids may be increased or decreased between wide limits by relatively slight changes in molecular structure so that pK is increased or decreased as interest may dictate. Or, acidic drugs such as salicylic acid and neoarsphenamine, which do not permeate membranes readily, may be made to permeate by conversion into esters or derivatives which do not form ionizable salts at body pH.

SUMMARY

1. The collodion membrane containing lecithin and cephalin is permeable to molecules, but not to ions.

2. Any factor, such as changes in pH, esterification, and salt formation, which tends to change the degree of ionization, likewise changes the rate of permeation of such membranes.

3. Those substances which penetrate the brain when injected intravenously also permeate these artificial membranes; and those which do not penetrate the brain do not permeate such membranes.

4. In view of this close parallelism it is suggested that cell membranes containing phospholipids separating blood stream and brain substance constitute the so-called blood-brain barrier.

REFERENCES

Weatherby, J. H.: Concerning the Mechanism of Membrane Semipermeability, J. Cell. & Comp. Physiol. 21: 1, 1943.

Friedemann, Ulrich: Blood-Brain Barrier, Physiol. Rev. 22: 125, 1942.

Friedemann, Ulrich, and Elkeles, A.: The Blood-Brain Barrier in Infectious Diseases, The Lancet 227: 775, 1934.

Friedemann, Ulrich: Further Investigations on the Blood-Brain Barrier, J. Immunol. 32: 97, 1937.

*Personal communication from Doctor P. S. Larson, Department of Pharmacology, Medical College of Virginia.

STUDIES ON THE DETOXICATION OF ORGANIC ARSENICAL COMPOUNDS

IV. The Protective Action of P-Aminobenzoic Acid Against Lethal Doses of Neoarsphenamine Without Inhibition of Trypanocidal Potency*

J. H. Sandground, Sc.D., and C. R. Hamilton, B.S.
Indianapolis, Ind.

INTRODUCTION

FROM extensive investigations in the field of bacterial metabolism, Woods[1] offered as a stimulating hypothesis the suggestion that it was by virtue of the chemical similarity obtaining between the two compounds that p-aminobenzoic acid acts as an inhibitor of the bacteriostatic property of sulfanilamide.

The structural relationship between certain phenylarsonate derivatives, such as atoxyl, tryparsamide, carbarsone, acetarsone, etc., on the one hand and p-aminobenzoic acid (hereafter abbreviated paba) on the other, is essentially comparable to that existing between paba and many of the sulfonamide compounds. In view of this analogy, we were impressed with the possibility that the trypanocidal potentials of the afore-mentioned arsenicals might be based on a mechanism similar in principle to the bacteriostatic mechanism postulated for the sulfonamides. To investigate this possibility an experiment was set up in which a group of rats infected with *Trypanosoma equiperdum* were treated with a massive (and usually lethal) dose of carbarsone to which a substantial quantity of paba had been added. Had there been an effective diminution of the trypanocidal action of carbarsone, these rats would have died in a few days from acute trypanosomiasis. Again, on the basis of our experience, we expected that they might succumb by about the fourth day from the toxic effect of an overdose of carbarsone. Actually the majority of the animals involved in this experiment developed few and only minor stigmas of arsenical poisoning. Furthermore they successfully overcame the hazard of death from trypanosomiasis.[2]

More interesting to us at the time than the failure of paba to inhibit the trypanocidal action of carbarsone was the entirely unsuspected possession by paba of a capacity to neutralize the lethal property of massive doses of carbarsone. This deduction was amply confirmed by further work not only with carbarsone, but also with such analogous pentavalent arsenicals as phenylarsonic acid, arsanilic acid, acetarsone, etc.[3] Later it was found that paba also has the capacity to protect rats against the lethal action of pentavalent phenyl stibonates, as exemplified by the German proprietory drug, stibosan.[4]

With a view to elucidating the mechanism underlying detoxication by paba, a large series of experiments were carried out. It was found[5] that a single administration of paba at the rate of 500 mg. per kilogram body weight can

*From the Lilly Research Laboratories.

Received for publication, July 8, 1943.

protect the lives of rats against several pentavalent arsenicals in quantities which considerably exceed their so-called "universal lethal dose" or $L.D._{100}$, e.g., 1500 mg. per kilogram of carbarsone, 450 mg. per kilogram of arsanilic acid, etc.

Of special interest and possibly of theoretical significance in connection with the mechanism of the detoxicating action was the finding that, whereas maximum beneficial effect is achieved when paba is injected simultaneously with, or even some time *prior to* the arsenical, when paba is introduced *subsequent to* the arsenical, its value is reduced almost in direct proportion to the interval that elapses.[6]

Unfettered by any theory as to the biochemical mechanism of the detoxicating process, our attention was directed early in the course of these investigations to determining broadly the potentialities and limitations of paba in conjunction with an array of other arsenical and antimonial compounds. Numerous experiments have been carried out with inorganic and organic arsenicals as represented by sodium arsenite, sodium cacodylate, mapharsen, arsphenamine, and neoarsphenamine, and the organic trivalent antimonial, tartar emetic. While paba was always used in substantial quantities as a detoxicant with the above-mentioned toxic substances, only in conjunction with neoarsphenamine were we able to obtain what seems to be unequivocal evidence of its capacity to interfere substantially with the lethal effect of large doses of the drug.

In view of the importance of neoarsphenamine in the treatment of syphilis, it is hoped that the publication of our experimental findings in this journal may be of interest.

EXPERIMENTAL PROCEDURE

Technically much greater difficulty is encountered in demonstrating clearcut detoxication of neoarsphenamine than is to be experienced in proving detoxication against such drugs as carbarsone. This difficulty arises from the fact that, in contrast with the chemical purity of the pentavalent arsenicals and the crystalloidal nature of their aqueous solutions, the trivalent arsenobenzene compounds, such as arsphenamine and neoarsphenamine, are physicochemical complexes which are difficult to prepare in the state of purity indicated by their theoretical formulas. The toxicity rating and therapeutic efficacy of different lots of these compounds can only be established by biological assays. Because of the variation inherent in individual animals, this procedure, besides being expensive in terms of time, involves large numbers of animals, standardized so far as feasible by breeding, sex, age, and weight. The results of assays usually require the application of statistical methods for their correct interpretation.[7] However, even when tests with a homogeneous population of rats are performed with exacting attention to detail (e.g., in special air-conditioned rooms), nebulous and uncontrollable meteorologic factors seem to influence the mortality rates.[8] At best, the results obtained from such toxicity assays are to be interpreted as relative (to a standard) and can validly be presented only in terms of the median lethal dose ($L.D._{50}$).[9, 10]

The demonstration of the possession by any substance of a detoxicating action against neoarsphenamine must necessarily involve the same principles of

procedure, unless the detoxicating action is of such a high order that notwithstanding adverse conditions attending the assays, the results of protection tests are so obvious that statistical "booby traps" do not represent a hazard to their interpretation.

Largely as a consequence of prevailing conditions of supply as well as of other fortuitous circumstances, the experiments the results of which are recorded below were conducted with rats of a very heterogeneous assortment obtained through several animal dealers. Three lots of neoarsphenamine were used, each of a different manufacturer's brand purchased on the open market. On testing, one of these lots, "C," turned out to have a much higher toxicity for rats than did the other two. After preliminary toxicity tests with the particular lot of arsenical, protection experiments involving from 5 to 20 rats with an approximately equal number of unprotected controls were usually set up on one or two dosage rates. The assays were often repeated at the same dose rate or, if need be, with higher or lower doses of neoarsphenamine.

Despite the several handicaps to uniformity of results, it has been our experience that the outcome of a protection experiment was not essentially different, whether the assay was performed with the conventional young male 100-gram rats, of highly inbred strain and starved the night before testing, or whether we utilized the more readily available "mongrel" rats weighing up to 175 grams. The only precaution we have observed was to see that the drug was administered in quantities proportional to the rat's weight and to match rats of various weights equally between "treated" and "control" groups.

Obviously in order to secure meaningful data with a limited number of rats, it is necessary that the outcome of a protection experiment should show a significantly higher mortality rate in the control series than in the protected group. This calls for a rather nicely adjusted neoarsphenamine dosage approaching the maximum tolerance level. To go appreciably beyond this point, as we have on several occasions learned to our consternation, may lead to the death of all or nearly all of the animals, both "protected" and "controls." Another difficulty that can arise is characterized by the death of a large proportion of rats within a very few hours or even minutes of injection. This may happen irrespective of the dosage of arsenical and any protective treatment that may have been attempted. Agonal symptoms in such cases are referable to an acute vascular disturbance. Deaths of this order are clearly distinguishable from the normal, truly toxicologic fatalities that develop in from one to three or more days following injection. Wright et al.[11] found that the colloidal (in contradiction to crystalloidal) fraction of solutions of neoarsphenamine was responsible for the so-called immediate toxic reactions in 85 per cent of their rats that suffered precipitate deaths from respiratory failure. In some respects deaths of this type (which are, apparently, beyond the protective control of paba) resemble the "nitritoid crisis" or acute vasoparetic reaction, an ever present element of risk in the arsenic treatment of syphilis. Rather late in the course of our investigations we found that the incidence of deaths of the immediate type with the resultant waste of material and effort could be considerably reduced by employing subcutaneous injection in lieu of the usual intravenous (caudal or saphenous vein) route of introducing the drug.

The freshly prepared aqueous solution in 2 to 5 per cent concentration is

injected either simultaneously with, or not more than fifteen minutes after, the administration of a fresh 10 per cent aqueous solution of the sodium salt of p-aminobenzoic acid. Our rats have been accommodated in large communal cages, provided with ordinary commercial "chow," and kept under observation for at least ten full days before termination of the test.

TABLE I

COLLATED RESULTS OF EXPERIMENTS

PROTECTIVE ACTION OF p-AMINOBENZOIC ACID ON RATS RECEIVING MASSIVE DOSES OF NEOARSPHENAMINE

NEOARSPHENAMINE BRAND	NEOARSPHENAMINE DOSE MG./KG.	DOSE OF PABA MG./KG.	PABA-TREATED RATS NO. USED	PABA-TREATED RATS NO. SURVIVED	NONPROTECTED CONTROLS NO. USED	NONPROTECTED CONTROLS NO. SURVIVED
"A"	250 intravenously	500 intraperitoneally	11	6	9	3
	260 intravenously	2500 orally	22	14	15	6
	275 intravenously	1200 intraperitoneally	28	25	10	3
	275 intravenously	2000 orally	17	13	20	4
"B"	240 intravenously	1000 intraperitoneally	10	9	10	6
	250 intravenously	1000 intraperitoneally	9	9	10	3
	260 intravenously	1000 intraperitoneally	6	5	6	3
	280 intravenously	1000 orally	7	5	7	2
"C"	175 subcutaneously	1000 orally 1000 intraperitoneally	10 50	4 37	21	1
	180 subcutaneously	1000 intraperitoneally	40	31	27	1
	215 subcutaneously	1000 intraperitoneally	20	12	20	0

DISCUSSION

That paba possesses in substantial measure the power of protecting rats against the lethal effect of high doses of neoarsphenamine is, we believe, conclusively demonstrated from the results of experiments tabulated above, even without resorting to mathematical treatment of the figures to fortify this inference. Had we summarily eliminated from consideration those animals that died shortly after injection (as is sometimes done[10] in official assays of neoarsphenamine toxicity), the resulting figures would have supported the contention with even greater force. While "death" as the end point of a biologic reaction is by its very finality the most rigid measure that can be employed for the demonstration of protection (or the absence of protection) against a potent poison, the "all or none" nature of the criteria used in expressing the results of an experiment does not permit the introduction of fractional values into the situation. Such "fractional values" as are brought out by comparing the survival time of all rats in treated and control groups, or by contrasting their general condition and toxic reactions, are by no means insignificant. This is well illustrated by the accompanying photograph of three rats taken on the final day of the experiment, the tenth day after treatment with 175 mg. per kg. neoarsphenamine "Brand C." The normal-looking animals, 1 and 3, have received paba and are typical representatives of the protected survivors. Rat 2 is the only animal living out of 10 controls. It has lost weight (from 100 grams to 58 grams), and for several days appeared moribund. The fact that this animal escaped death, even though by a narrow margin, leads to its survival being credited in the protocol of results with the same unit value as any one of the paba-treated rats, e.g., 1 and 3, which have grown from 100 grams to 138 and

141 grams respectively without ever having been greatly indisposed by their arsenical medication. It is interesting to note in this connection that in our series of experiments the protective capacity of paba received its most rigorous test and appears to be most unequivocally demonstrated when a batch of neoarsphenamine (Brand C) of higher than usual toxicity was employed.

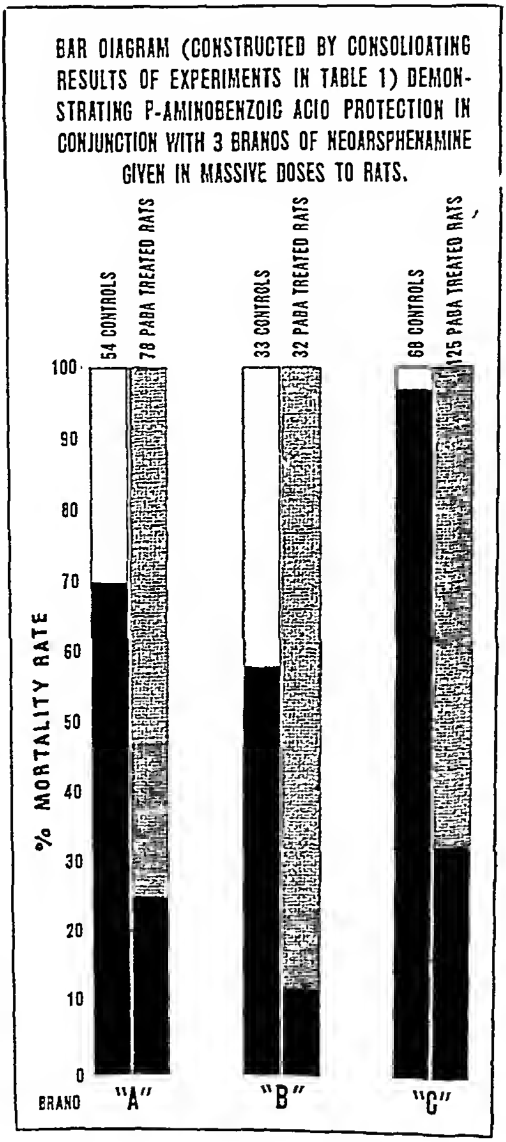


Fig. 1.

The clinical implication of the discovery that in rats paba has definite detoxicating value in conjunction with neoarsphenamine is difficult to evaluate without a critical trial. The subject is of sufficient importance to warrant a more extended consideration of its theoretical aspects than present space will permit. If paba is found to exercise a protective influence in human medication with neoarsphenamine, the question of its possible interference with the latter's treponemicidal activity will determine its clinical usefulness. In this connection,

the fact that in numerous experiments performed by us with *Trypanosoma equiperdum* paba in relatively large quantities was not found to diminish the parasiticidal value of neoarsphenamine may be of inferential significance.* This is to be contrasted with the experience of Durel,[13] who found that while solution of novarsenobenzol (=neoarsphenamine) in ascorbic acid appeared to reduce the toxicity of the arsenical in mice, the death rate from *Trypanosoma brucei* following treatment with a uniform dose of novarsenobillon was directly proportional to the amount of ascorbic acid contained in the mixture.† Loss of therapeutic efficacy with high doses of vitamin C in conjunction with arsenotherapy has also been suggested in clinical observations by Cormia.[14]

Fig. 2.—Photograph of rats treated with 175 mg. per kg. neoarsphenamine. Rat 2 is the sole survivor of 8 controls (see text). Rats 1 and 3 are typical of the 16 survivors out of 20 animals that received a simultaneous injection of *p*-aminobenzoic acid.

It should not be necessary to remark that a great chasm separates experience acquired with animals in the experimental laboratory from that which may be experienced in the clinic. Differences in the results obtained sometimes reflect fundamental differences in the metabolic processes of the animal species as reflected in their methods of disposing of certain chemical substances, e.g., benzoic acid. Frequently, however, the differences may be ascribed to the lack of parallelism between the conditions governing the laboratory experiment and those which are feasible when dealing with human lives. In advance it may be well to remark that in rat experiments the evidence of "paba" detoxication is best brought out by using doses of neoarsphenamine of a magnitude that has no parallel in the clinic unless it be in conjunction with the intensive arsenotherapy of syphilis. No evidence has been disclosed that would warrant the assumption that paba can act as an effective antidote against a toxic condition induced by the *prior* administration of arsenicals or that the concomitant administration of paba will eliminate the danger of the "nitritoid crisis" or counteract an "acquired sensitiveness" to arsenicals.

SUMMARY

Proceeding from a knowledge of the capacity of p-aminobenzoic acid to protect rats against the "certain lethal dose" ($L.D._{100+}$) of various pentavalent

*This inference is strengthened by the incidental observation of Osgood[12] who succinctly remarks "p-aminobenzoic acid does not interfere with the action of neoarsphenamine as it does with the action of the drugs of the sulfanilamide group."

†Incidentally, it may be recorded that in several parallel experiments we have not found ascorbic acid to have detoxicating power comparable with that of paba.

arsenical and antimonial compounds, a large series of experiments have been performed to determine the scope of paba protection in conjunction with several trivalent arsenical and antimonial drugs.

It appears that paba has no detoxicating capacity against inorganic arsenous acid; the arsenoxide mapharsen; the arsenobenzene arsphenamine; or the trivalent organic antimonial tartar emetic.

Against neoarsphenamine, protection experiments with a large series of unselected rats are reported in this paper. The results show a very substantial reduction in the mortality rate without a concomitant inhibition of its parasiticidal potency as measured by its effect on trypanosomiasis in the rat.

The clinical implication of these findings, especially in connection with the intensive treatment of syphilis, warrant further investigation.

REFERENCES

1. Woods, D. D.: The Relation of p-Aminobenzoic Acid to the Mechanism of the Action of Sulphanilamide, Brit. J. Exper. Path. 21: 74, 1940.
2. Sandground, J. H.: p-Aminobenzoic Acid Detoxication of Carbarsone (p-Carbamino Phenyl Arsonic Acid) and Certain Other Pentavalent Phenyl Arsonates Administered in Massive Doses to Rats, Science 97: 73, 1943.
3. Sandground, J. H., and Hamilton, C. R.: Studies on the Detoxication of Organic Arsenical Compounds. I. Detoxication by Means of p-Aminobenzoic Acid of Certain Pentavalent Arsenical Drugs Given in Massive Doses to Rats, J. Pharmacol. & Exper. Therap. 78: 109, 1943.
4. Sandground, J. H.: Detoxication of a Substituted Phenyl Stibonate in the Rat by Administration of p-Aminobenzoic Acid, Proc. Soc. Exper. Biol. & Med. 52: 188, 1943.
5. Sandgronnd, J. H., and Hamilton, C. R.: Studies on the Detoxication of Organic Arsenical Compounds. II. Correlation of the Qnantity of p-Aminobenzoic Acid Required to Protect Rats Against High Doses of Carbarsone and Arsanilic Acid, J. Pharmacol. & Exper. Therap. 78: 203, 1943.
6. Sandground, J. H.: Studies on the Detoxication of Organic Arsenical Compounds. III. The Time Factor Influencing p-Aminobenzoate Protection of Rats Receiving Lethal Doses of Phenyl Arsonates, J. Pharmacol. & Exper. Therap. 78: 209, 1943.
7. Morrell, C. A., Chapman, C. W., and Allmark, M. G.: A Study of the Effect of Age, Weight, Sex, and Dose on Length of Survival of Albino Rats Infected With *Trypanosoma equiperdum* With Particular Reference to Variations in Host, Am. J. Hyg. 25: 232, 1937.
8. Nedsel, J. A.: Daily Variations in Toxicity of Neoarsphenamine in White Rats, J. Lab. & Clin. Med. 27: 720, 1942.
9. Trevan, J. W.: The Error of Determination of Toxicity, Proc. Roy. Soc., London, ser. B. 101: 483, 1927.
10. Hawking, F.: The Standardization of the Therapeutic Potency of Neoarsphenamine and Sulpharsphenamine, Quart. J. Pharm. & Pharmacol. 16: 13, 1943.
11. Wright, H. N., Biedermann, A., Hanssen, E., and Cooper, C. I.: Physico-Chemical Properties of the Arsphenamines in Relation to Toxicity and Therapeutic Efficiency, J. Pharmacol. & Exper. Therap. 73: 12, 1941.
12. Osgood, E. E.: Noarsphenamine Therapy of Bacterial Infections, Arch. Int. Med. 69: 746, 1942.
13. Durel, M. P.: Influence de la vitamin C sur la toxicité et l' activité du novarsenobenzol chez la souris, Bull. Soc. franç. de dermat. et syph. 44: 1077, 1937.
14. Cormia, F. F.: Postarsphenamine Dermatitis: The Relation of Vitamin C to Production of Arsphenamine Sensitiveness and Its Use as an Adjunct to Further Arsphenamine Therapy, J. Invest. Dermat. 4: 81, 1941.

CLINICAL CHEMISTRY

ABSORPTION, EXCRETION, AND DISTRIBUTION OF SULFAMETHAZINE (2-SULFANILAMIDO-4-6-DIMETHYL-PYRIMIDINE) IN MAN*

JOHN K. CLARK, M.D., FRANKLIN D. MURPHY, M.D., AND
HARRISON F. FLIPPIN, M.D., PHILADELPHIA, PA.

THERE has been a marked improvement in the therapeutic efficacy and lack of toxicity in the series of sulfonamides that has been introduced for clinical use since sulfanilamide. Among the more recent of these compounds, sulfamerazine (2-sulfanilamido-4-methyl-pyrimidine) and sulfamethazine (2- sulfanilamido-4-6-dimethyl-pyrimidine) have warranted clinical trial because of their therapeutic effectiveness against various experimental infections and the relatively high solubility of their acetylated forms.

Since the intelligent use of these drugs requires an understanding of their behavior when administered to man, we have investigated their fate in a group of patients. In a recent paper,[1] we reviewed the experimental data pertaining to sulfamerazine and presented observations dealing with its behavior in man; this suggested that sulfamerazine is pharmacologically satisfactory. At this time we are reporting the results of a similar study on sulfamethazine† and have made a comparison of the two drugs on the basis of their absorption, excretion, and distribution in human beings.

Sulfamethazine (2-sulfanilamido, 4-6-dimethyl-pyrimidine), described by several groups of investigators,[2-4] is a dimethyl homologue of sulfadiazine. The therapeutic effectiveness of sulfamethazine against experimental infection has been investigated by Rose, Martin, and Bevan[4] and found to be highly active against pneumococcal and streptococcal infections in mice. Pharmacologic studies, both in mice and human beings,[4] indicate that the drug is freely and rapidly absorbed from the gastrointestinal tract and it is stated to be slowly eliminated from the blood stream. Furthermore, it was suggested that, because of its high solubility, sulfamethazine is unlikely to cause renal damage.[4] Preliminary clinical trials with the compound have shown that it is highly effective in pneumococcal, meningococcal, and gonococcal infections and is less toxic than is sulfapyridine.[5, 6]

The methods used in this investigation were the same as those employed in the sulfamerazine study.[1]

*From the Department of Medicine and the William Pepper Laboratory of the Hospital of the University of Pennsylvania.

This work was aided by a grant from the Sharp and Dohme Laboratories, Glenolden, Pa.
Received for publication, July 13, 1943.

†The sulfamethazine used in this study was supplied us by Sharp and Dohme Laboratories, Glenolden, Pa.

RESULTS

A. Absorption.—

1. *Single Oral Dose.*—Nine patients (Nos. 1 to 9, Table I) were given a single 3 Gm. dose of the drug. At the end of 2 hours the average concentration of free sulfamethazine in the serum was 11.1 mg. per 100 c.c., and, after 4 hours, the average concentration reached a peak of 12.0 mg. per 100 c.c. Thereafter, the level began to fall, and at the end of 12 and 24 hours it averaged 6.1 and 2.2 mg. per 100 c.c. respectively. Acetylation of the drug varied. At the end of 6 hours an average of 21.6 per cent of the total drug was in the acetylated form; at 12 hours, 37.1 per cent, and after 24 hours, 48.8 per cent.

2. *Single Intravenous Dose.*—Two patients (Nos. 10 and 11, Table I) were each given 3 Gm. of sulfamethazine sodium in 5 per cent solution in sterile distilled water intravenously. Within one-half hour the average level of free drug was 15.3 mg. per 100 c.c.; in 2 hours the concentration averaged 11.4 mg.; after 4 hours, 9.5 mg.; and, at the end of 24 hours, 1.9 mg. per 100 c.c.

3. *Single Subcutaneous Dose.*—Two patients (Nos. 12 and 13, Table I) were each given 3 Gm. of sulfamethazine sodium, 0.5 per cent concentration in 0.85 per cent sodium chloride solution, subcutaneously. The average concentration of free drug reached a peak of 8.5 mg. per 100 c.c. at 2 hours, within 4 hours the level was 7.1 mg., after 6 hours, 5.9 mg., and at the end of 24 hours, 0.5 mg. per 100 c.c. Acetylation of the drug after parenteral administration very closely paralleled that found with oral administration.

4. *Multiple Dosage.*—Twelve patients, suffering from several diseases, including pneumococcal pneumonia, meningococcal septicemia, puerperal sepsis, postoperative pneumonia, were treated with sulfamethazine for varying periods of time and with different dosage schedules. In general, they were given 2 to 4 Gm. by mouth, followed by 1 Gm. every 4 to 6 hours. Serum levels varied widely from 1.0 to 23.0 mg. per 100 c.c. of the free drug with an average acetylation of 38.3 per cent (range 0.8 to 83.5 per cent). While serum concentrations of sulfamethazine varied greatly from person to person, they were constant for the same individual.

B. Excretion.—

Urinary excretion of the drug was measured (Table I). After the oral administration of a single 3 Gm. dose, from 17.1 to 55 per cent (average 40.1 per cent) was recovered in the first 12 hours; by the end of 24 hours from 40.8 to 90 per cent (average 71.8 per cent) had been excreted, and, at the end of 48 hours from 67.6 to 98.8 per cent (average 86.2 per cent) of the ingested drug was recovered from the urine. During the first 24 hours an average of 70.9 per cent of the excreted drug was in the acetylated form, and, after 48 hours, 77.3 per cent was acetylated drug. Similar results were obtained when the drug was given parenterally (Table I).

C. Distribution.—

1. *Cerebrospinal Fluid.*—Four patients (Nos. 14, 15, 16, and 17, Table II) were given the drug by mouth, and cerebrospinal fluid and blood specimens were obtained at varying intervals. Six hours after the administration of 3 Gm. of sulfamethazine, the ratio of the cerebrospinal fluid level of free drug to that

TABLE I

FATE OF SULFAMETHAZINE ADMINISTERED BY VARIOUS ROUTES

ABSORPTION AND EXCRETION OF SINGLE ORAL DOSE (3 GM.) OF SULFAMETHAZINE

			SERUM			URINE						
						SULFAMETHAZINE			CUMULATIVE EXCRETION (%)			
NO.	PATIENT AGE (YEARS) WT. (LBS.)	TIME (HRS.)	FREE (MG. PER 100 C.C.)	TOTAL (MG. PER 100 C.C.)	ACETYLATED (%)	FREE (MG. PER 100 C.C.)	TOTAL (MG. PER 100 C.C.)	ACETYLATED (%)	FREE	TOTAL	URINE VOLUME (C.C.)
1	R. P.	2	9.0	9.2	2.2						
	36	4	11.2	12.0	6.7						
	160	6	11.3	12.9	12.4						
		12	6.5	9.0	27.8	83.0	130.0	36.2	29.7	32.5	750
		24	2.4	3.5	31.4	74.3	145.0	48.8	35.6	54.0	600
		48				17.5	52.5	66.7	42.2	80.4	1135
2	J. S.	2	15.8	15.8	0						
	43	4	14.8	16.1	8.1						
	130	6	14.0	15.7	10.8						
		12	9.5	11.4	16.7	111.4	244.5	44.1	21.2	35.8	440
		24	3.6	4.9	26.1	117.0	239.5	51.2	36.4	67.1	390
		48				17.6	67.3	73.8	39.7	79.2	510
3	A. P.	2	2.5	2.5	0						
	17	4	7.5	7.5	0						
	120	6	12.6	13.1	3.9						
		12	11.4	12.0	5.0	28.6	45.0	36.5	10.9	17.1	1140
		24	6.0	8.2	26.8	73.1	148.5	50.8	22.2	40.8	480
		48				23.8	66.2	64.1	30.5	67.6	1040
4	K.	2	17.0	18.3	7.2						
		4	15.0	15.7	1.5						
		6	14.1	15.0	6.0						
		12	8.0	10.0	20.0	30.3	53.0	42.8	19.2	33.6	1900
		24	3.8	3.5		30.8	90.0	65.8	33.3	74.8	1375
		48				10.1	40.0	74.8	39.3	98.9	1800
5	O.	2	8.5	10.6	19.8						
		4	11.9	16.1	25.7						
		6	11.7	17.7	33.9						
		12	5.3	12.8	58.7	41.5	170.5	73.9	10.1	40.9	720
		24	1.5	4.7	68.1	12.0	70.0	82.9	15.6	71.5	1310
		48				4.3	25.0	82.8	18.7	89.9	2200
6	M.	2	11.5	15.4	25.3						
	35	4	13.8	18.7	26.2						
	140	6	10.5	17.5	40.4						
		12	3.1	9.0	65.6	128.3	530.0	75.8	13.3	54.7	310
		24	1.0	3.8	73.7	70.3	540.0	86.8	18.0	90.7	200
		48				15.0	98.0	84.7	19.1	97.6	210

7	R. C.	2	12.7	18.0	29.4						
	20	4	9.8	12.1	19.0						
	140	6	4.5	8.9	49.4						
		12	.5	4.5	88.9	60.0	445.0	86.5	6.4	47.5	320
		24	.3	2.0	85.0	5	135.0	96.3	7.8	84.4	820
		48				1.3	9.3	86.0	15.5	89.9	1775
8	F. W.	2	16.2	17.2	5.8						
	35	4	12.9	14.0	7.9						
	160	6	12.5	13.9	10.1						
		12	6.5	8.5	23.5	42.5	90.5	53.6	20.9	45.4	1440
		24	1.5	4.5	66.7	59.0	159.5	63.0	29.9	70.0	500
		48				12.1	43.1	71.9	43.8	75.0	345
9	L. S.	2	6.3	7.3	13.7						
	30	4	11.0	14.6	24.6						
	150	6	9.6	13.9	30.9						
		12	3.8	10.3	63.1	63.0	412.5	84.6	8.4	55.0	400
		24	0.1	3.5	97.1	42.5	455.0	90.7	13.0	90.0	235
		48				4.0	41.3	90.3	13.6	97.2	475
INTRAVENOUS INJECTION OF SINGLE 3 GM. DOSE OF SULFAMETHAZINE SODIUM											
10	R. B.	½	13.0	14.5	10.4						
	35	2	7.9	11.4	30.7						
	175	4	5.5	8.3	34.9						
		6	4.5	6.9	34.8						
		12	.8	2.8	71.4	27.5	107.5	74.4		41.2	1150
		24	.3	1.4	21.4	12.5	108.8	88.4		61.1	550
		48				3.2	18.4	83.1		71	1550
11	S. G.	½	17.6	17.8	1.1						
	40	2	14.9	15.9	6.3						
	160	4	13.5	13.8	2.2						
		6	11.3	12.3	8.1						
		12	6.5	7.1	8.4	22.5	47.5	54.7		53.8	3250
		24	3.5	4.0	12.5	52.8	77.5	31.8		74.5	800
		48				14.0	25.7	45.5		75	1665
SUBCUTANEOUS INFUSION OF SINGLE 3 GM. DOSE OF SULFAMETHAZINE SODIUM											
12	R. E.	2	9.2	9.3	1.2						
	19	4	9.3	9.8	5.1						
	130	6	8.3	8.6	3.5						
		12	4.9	6.4	23.4	21.8	46.0	52.6			1565
		24	0.7	3.5	80.0	29.5	106.0	72.2			850
		48				6.0	27.2	77.9		72	1900
13	S. H.	2	7.8	11.5	32.2						
	40	4	4.9	9.9	50.5						
	165	6	3.5	8.0	56.3						
		12	0.5	3.0	83.3	25.0	113.5	77.9			1365
		24	0.1	0.5	80.0	5.0	52.5	94.1			675
		48				0.6	6.0	90.0		67	1660

in the serum was 0.04 (No. 14). After 12 hours, following a similar dose of the drug, the ratio was 0.58 (No. 15). Two patients were given multiple doses of sulfamethazine, and after 24 hours (No. 16) and 11 days (No. 17) the ratios were 0.33 and 0.29 respectively.

2. *Pleural and Ascitic Fluid.*—In three patients (Nos. 18, 19, and 20, Table II) who received sulfamethazine pleural or ascitic fluid and blood samples were taken at varying intervals. Ratios of 0.86 and 0.53 were obtained in pleural fluid 12 hours (No. 18) and 4 days (No. 19) following 3 and 20 Gm. of the drug respectively. In one patient (No. 20), after 20 Gm. of the drug, the ratio of 0.51 in ascitic fluid was obtained.

3. *Erythrocytes.*—Simultaneous determinations of sulfamethazine concentration in serum and whole blood were performed in a single patient (Table III). The results in this instance show that the drug penetrates the red blood cells.

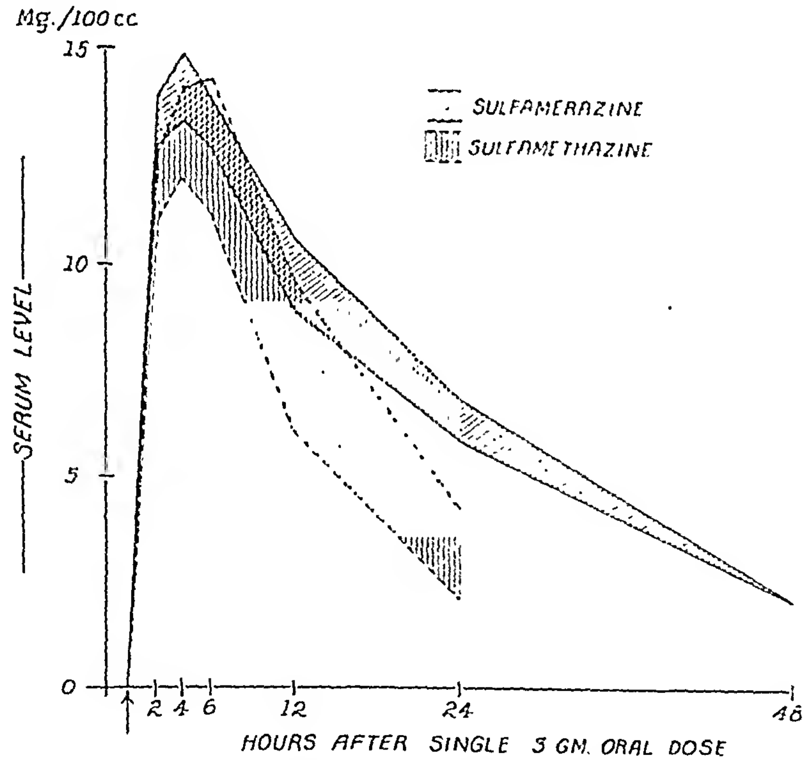


Fig. 1.—Comparison of average serum concentrations obtained in 9 subjects receiving 3 Gm. of sulfamethazine with those obtained in 8 subjects receiving a similar amount of sulfamerazine. In each curve the lower line indicates free drug and the upper line, total drug.

DISCUSSION

Comparison of these data on sulfamethazine with recent studies in this laboratory on sulfamerazine[1] indicates that the two drugs are absorbed from the gastrointestinal tract with equal rapidity (Fig. 1). The rate of excretion of both free and acetyl-sulfamethazine is greater than that found with sulfamerazine and its acetyl derivative (Fig. 2). The two drugs diffuse readily into various body tissues and tissue fluids. In view of the greater solubility of

TABLE II

FREE SULFAMETHAZINE CONCENTRATION IN VARIOUS BODY FLUIDS

BODY FLUID	NO.	PATIENT	HOURS AFTER INITIAL DOSE	NO. OF DOSES	TOTAL DOSE (GM.)	SERUM (MG. PER 100 C.C.)	BODY FLUID (MG. PER 100 C.C.)	CONCENTRATION RATIO (BODY FLUID/ SERUM)
Cerebrospinal Fluid	14	R. G.	6	1	3	7.5	0.3	0.04
	15	R. S.	12	1	3	1.9	1.1	0.58
	16	M. B.	24	4	7	9.5	3.1	0.33
	17	M. L.	11 days	39	41	21.9	6.3	0.29
Pleural Fluid	18	W. B.	12	1	3	2.8	2.4	0.86
	19	B. S.	4 days	18	20	13.4	7.1	0.53
Ascitic Fluid	20	B. S.	4 days	18	20	13.4	6.8	0.51

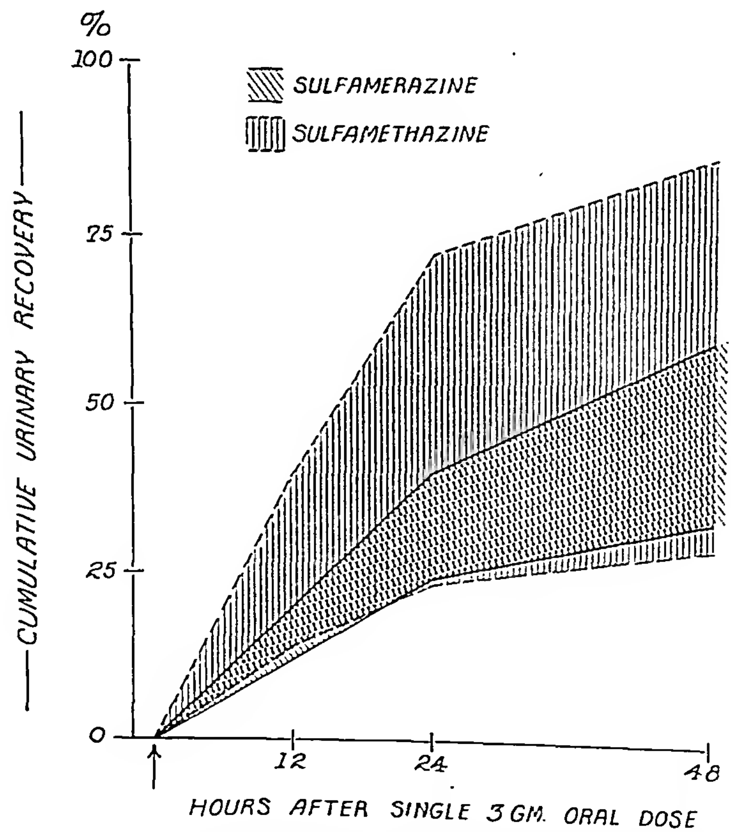


Fig. 2.—Comparison of average cumulative urinary excretions obtained in 9 subjects receiving 3 Gm. of sulfamethazine with those obtained in 8 subjects receiving a similar amount of sulfamerazine. In each curve the lower line indicates free drug and the upper line, total drug.

sulfamethazine,[3] the incidence of urinary complications may be less than with sulfamerazine. Because of its rapid excretion and high acetylation, both in serum and urine, sulfamethazine appears to be less satisfactory from a pharmacologic viewpoint than sulfamerazine. The above findings are in keeping with those recently reported by Welch and his associates.[5]

TABLE III

DISTRIBUTION OF SULFAMETHAZINE BETWEEN SERUM AND RED BLOOD CELLS
SULFAMETHAZINE CONCENTRATION IN MG. PER 100 C.C.

NO.	PATIENT	DAY OF DRUG	WHOLE BLOOD FREE	WHOLE BLOOD TOTAL	SERUM FREE	SERUM TOTAL	CELLS (CALCULATED) FREE	CELLS (CALCULATED) TOTAL	PACKED CELL VOLUME (%)
21	M. L.	11	15.5	16.3	21.9	23.9	6.0	5.0	40

SUMMARY

Data are presented concerning the absorption, excretion, and distribution of sulfamethazine in humans. They show: (1) that sulfamethazine is readily absorbed from the gastrointestinal tract; (2) that it is rapidly excreted in the urine; and (3) it readily diffuses into pleural, ascitic, and cerebrospinal fluids and into the red blood cells. Comparison with the behavior of sulfamerazine shows that higher blood concentrations are sustained longer with sulfamerazine than after similar amounts of sulfamethazine.

REFERENCES

1. Murphy, F. D., Clark, J. K., and Flippin, H. F.: Studies on 2-Sulfanilamido-4-Methyl-Pyrimidine (Sulfamerazine, Sulfamethyldiazine) in Man. I. Absorption, Distribution, and Excretion, Am. J. M. Sc. 205: 717, 1943.
2. Caldwell, W. T., Kornfeld, E. C., and Donnell, C. K.: Substituted 2-Sulfanilamidopyrimidines, J. Am. Chem. Soc. 63: 2188, 1941.
3. Roblin, R. O., Jr., Winnek, P. S., and English, J. P.: Studies in Chemotherapy; Sulfanilamidopyrimidines, J. Am. Chem. Soc. 64: 567, 1942.
4. Rose, F. L., Martin, A. R., and Bevan, H. G. L.: Sulphamethazine 2-4-AminoBenzenesulphonylamino-4:6-dimethylpyrimidine) A New Heterocyclic Derivative of Sulphanilamide, J. Pharmacol. & Exper. Therap. 77: 127, 1943.
5. McCartney, D. W., Smith, G. S., Luxton, R. W., Ramsay, W. A., and Goldman, J.: Sulphamethazine: Clinical Trial of a New Sulphonamide, Lancet 1: 639, 1942.
6. Jennings, P. A., and Patterson, W. D.: Sulphamethazine. Clinical Trials in Children, Lancet 2: 308, 1942.
7. Clark, J. K., Flippin, H. F., and Murphy, F. D.: Studies on 2-Sulfanilamido-4-Methyl-Pyrimidine (Sulfamerazine, Sulfamethyldiazine) in Man. II. Toxic Manifestations, Am. J. M. Sc. 205: 846, 1943.
8. Welch, A. D., Mattis, P. A., Latven, A. R., Benson, W. M., and Shiels, E. H.: Sulfamerizine (2-Sulfanilamido-4-methylpyrimidine). I. A Comparison of Sulfamerizine with Sulfadiazine on the Basis of Absorption, Excretion, and Toxicity, J. Pharmacol. & Exper. Therap. 77: 357, 1943.

STUDIES IN THE PHARMACOLOGY OF MERCURY

III. Histochemical Demonstration and Differentiation of Metallic Mercury, Mercurous Mercury, and Mercuric Mercury*

W. C. Hand, B.S., B. B. Edwards, B.S., and E. R. Caley, Ph.D.†
New Brunswick, N. J.

Mercury and its compounds have been used for many years in the prevention and treatment of syphilis; yet a review of the literature discloses little material on methods for observing the valency changes which may take place during and after the absorption of mercury and its derivatives from the skin and other depots of administration. The development and utilization of differential methods for demonstrating mercury in the organs of the body should serve to extend our knowledge of the pharmacology of this element and perhaps lead ultimately to more efficacious clinical preparations. In this paper we present some new histochemical methods together with a review and extension of certain older ones.

CHEMICAL CONSIDERATIONS

General.—It is self-evident that a histochemical reagent should react in a characteristic manner with the substance to be detected. As Christeller[1] points out, this is easy in the case of iron, since there are a number of brilliant-hued specific chemical reactions. In spot tests[2] mercury likewise gives fairly specific color reactions with such reagents as diphenylcarbazide and dithizone. Our experiments with the above reagents on sections known to contain mercury compounds were disappointing. The color was either too faint or too evanescent. Precipitation with sulfides according to the procedures of Justus[3] and of Almkvist[4] also gave inconclusive results. The particle size of mercury sulfides formed in tissues may be extremely small, so that it is difficult to differentiate them from hemosiderin and other pigments. The use of iodine and iodide proved fruitless, as previously noted by Christeller because the entire section is stained. We ultimately confirmed the conclusion previously reached by Lombardo[5] and by Christeller that the reduction of mercury in the tissues to the metallic form is the simplest and most reliable histochemical procedure.

Metallic Mercury.—As has been pointed out by Zwick[6] and by Emich,[7] the microscopic appearance of finely divided mercury is highly characteristic. The particles are spherical, black, and nonrefractile. When sufficiently large, an image of the lens of the objective is reflected from their centers. Their very roundness makes them subject to ready displacement, however, and the section is best cut rather thick (15 microns) if free mercury is suspected, in order that a comparatively undisturbed inner zone may be available for inspection.

Zwick demonstrated that metallic mercury is present in the subcutaneous

*Part I of Studies in the Pharmacology of Mercury. J. Am. Pharm. A. (Scient. Ed.) 32: 255, 1943. Part II, in press.

†Contribution of Wallace Laboratories, Inc., New Brunswick, N. J.

Received for publication, August 5, 1943.

tissues after innuction of the element in a salve vehicle. We have confirmed this observation. He pointed out that the globules react with components of tissue fluids and lose their spherical shape. According to Christeller, they dissolve completely in tincture of iodine and this characteristic reaction may be observed under the microscope. We have observed further that solutions of chlorauric acid react with the minute spheres, causing at first a loss of gloss and a roughening of surface; this is followed by a gradual disintegration of the spheres as the gold amalgam forms. These changes were observed microscopically in sections of tissue. The above-mentioned characteristic microscopic appearance and microchemical behavior permit positive identification of the metal.

Mercurous Mercury.—Compounds containing mercurous mercury are usually colorless. Most mercurous salts, for example, the acetate, combine or mix with blood and other protein systems without the formation of opaque or colored bodies. For histochemical purposes, it is accordingly necessary to render the compounds visible. This can be done, even in the presence of mercuric mercury, by employing a reagent with a selective reducing action, so that only the mercurous mercury is reduced to the metal. Thioglycollic acid exhibits this differential selective reducing effect. In test tube experiments calomel is reduced immediately. Under the microscope, a suspension of calomel crystals is transformed almost instantaneously to a dispersion of metallic globules, when a drop of thioglycollic acid is introduced under the cover slip.

Mercuric salts are not so reduced. They react with thioglycollic acid to form compounds which appear as colorless to yellow crystals. We have not attempted to determine the precise nature of these compounds as they are found in histochemical reactions. It has been reported, however, by Claesson[8] that mercuric compounds react with thioglycollic acid to form either mercurithioglycollic acid ($Hg[S.CH_2COOH]_2$) or mercuric thioglycollate ($Hg[S.CH_2COO]_2Hg$), according to conditions. It is likely, that in the histochemical testing where an excess of reagent is normally present only the first of these two compounds is formed in appreciable amount. The presence of crystals of mercurithioglycollic acid does not interfere with the histochemical interpretation of the sections. Although the appearance of the crystals is rather characteristic, we do not recommend that they be regarded as an index of the mercuric mercury present. This reservation is based on the fact that they may dissolve and recrystallize at a point remote from the original site of the mercuric compound.

Tissue sections which contain mercurous mercury exhibit a prompt formation of mercury when a thioglycollic acid solution is introduced under the cover slip. The gradual formation of these globules can be followed visually. Should mercuric mercury also be present in appreciable amount, characteristic yellowish crystals begin to form after about five minutes.

Mercuric Mercury.—It was established by Lombardo and confirmed by Christeller that mercury is precipitated from tissues by stannous chloride solutions. This is a well-known chemical phenomenon which can be demonstrated readily in the test tube. Stannous chloride solutions reduce both mercurous and mercuric salts (including the thioglycollic acid mercury salts) to free mercury. It is thus possible, first, to examine a section for free mercury, then, to detect the mercurous mercury with thioglycollic acid, and, finally, to

detect the remaining mercuric mercury with stannous chloride. In actual practice, however, we prefer to treat three separate serial sections with the several reagents as described below. Sodium stannite solutions also are effective, but they macerate the tissue.

SUITABLE REAGENTS

General.—The reagents used in these studies were developed by a process of trial and error. It is not claimed, nor maintained, that they represent the best possible embodiment of the reducing agents. They have been tested, however, on thousands of sections from more than fifty animals including rabbits, guinea pigs and mice, with satisfactory results. In general, no counterstains have been employed. In our experience counterstains tend to displace the mercury globules.

Metallic Mercury.—As has been noted elsewhere, it is not necessary to use a reagent to demonstrate the presence of free mercury. Inasmuch as this work has been done almost exclusively with frozen sections, however, a clearing agent is employed. Its formula is as follows:

Formula I:	Tartaric acid	5 Gm.
	Glycerin	100 milliliters
Preparation:	Add the acid to the glycerin and agitate. The crystals will dissolve overnight.	

The above solution removes hemosiderin deposits and provides sections which exhibit adequate cellular detail without counterstaining.

As a confirmatory test to dissolve the mercury, official tincture of iodine may be used.

Formula II:	Iodine	70 Gm.
	Potassium Iodide	50 Gm.
	Distilled Water	50 Gm.
	Ethyl Alcohol, 95 per cent	To make 1000 milliliters
Preparation:	Dissolve potassium iodide in the water, add the iodine and agitate until solution is effected. Then add sufficient alcohol to make 1000 milliliters and mix thoroughly.	

The gold amalgam reaction is sometimes useful in fresh frozen sections of the skin because it does not stain initially as intensely as does iodine. This test is occasionally useful when confusion is experienced with skin pigments.

Formula III:	Chlorauric acid	1 gram
	Distilled water	99 milliliters
Preparation:	Dissolve the chlorauric acid in the distilled water in the cold with stirring. This reagent should be stored in a dark bottle.	

Mercurous Mercury.—The following solution of thioglycollic acid has been found satisfactory:

Formula IV:	Thioglycollic acid	1 milliliter
	Glycerin	9 milliliters
Preparation:	The above are completely miscible.	

Mercuric Mercury.—Stannous chloride reagent of the following composition may be stabilized by adding a few grams of metallic tin to the final solution which should be kept in a well-stoppered bottle.

Formula V:	Stannous chloride	5 Gm.
	Tartaric acid	5 Gm.
	Glycerin	100 milliliters
Preparation:	Combine and heat until the solution is clear.	

HISTOLOGIC TECHNIQUE

Throughout this study, every attempt was made to avoid artefactual changes in the tissues. The extended fixation of tissue advocated by Christeller and Lombardo may result, as the former author noted, in a *leaching out* of mercury compounds. Also, the immersion of whole sections permits diffusion of mercury compounds within the specimen with the resultant introduction of unpredictable inaccuracies in the final readings. Furthermore, the usual histologic fixations may similarly contribute an unknown artefactual change in the specimens. As a matter of fact, Christeller observed that formalin and alcohol decreased his yield. In work with frozen sections of material previously fixed in alcohol or formalin, we noted, histochemically, that our reagents usually produced a precipitate on the exposed surface of the sections only. For these reasons and in the interests of more rapid work, unfixed frozen sections were employed in our experiments.

In our standard practice the sections are taken immediately after sacrificing the animals, because experience showed a loss of clarity in cellular detail if the specimens were left at room temperature more than thirty minutes after death. It is believed that the small interval between sacrifice of the animal and completion of the finished section contributed in no small measure to the reproducibility of the findings.

The procedure is the same with Reagents I, IV, and V. The fresh section is placed on the freezing microtome over a piece of damp tissue paper which aids in the adherence of the specimen. Sections of about 15 microns are then cut, placed on a clean slide by means of a brush, and left uncovered until dry. This is necessary in case of Reagent V, to avoid precipitating stannous hydroxide. When dry, one drop of the reagent is added, a cover slip is placed over it, and the excess of the reagent blotted away. Then the edges of the cover slip are sealed with commercial gold size.* It is imperative that the cover slips be well sealed because of the pronounced humectant and creeping properties of the glycerin. The entire sectioning, mounting, and testing with reagent can be finished within ten minutes. The reagents act promptly and the slides are ready for reading ten minutes after they are made. Slides prepared with Reagent I, which is routinely used as a control, as well as those prepared with Reagent V, usually remain unchanged for at least two weeks.

It may be noted here that tissue sections which contain both mercurous and mercuric ions can give a misleading impression if they are read too long after the application of the thioglycollic acid reagent (IV). The reason for this is that free mercury will react with mercuric mercury in a complex oxidation-reduction

*Commercial gold size is the type of adhesive used for applying gold foil to plate glass.

system. Additional free mercury is liberated during the process. This reaction is slow, in the sections, so that no visible change is observed over a period of several hours. Twenty-four hours later, however, the globules may have become more numerous and larger. One may take advantage of this reaction, if one wishes to establish the presence or absence of submicroscopic quantities of mercurous mercury in a tissue known to contain mercuric ions. If there has been any mercurous ion present it will have manifested itself as mercury after several days because of the reaction described above. Under ordinary circumstances, we recommend that slides treated with reagent IV be photographed or read within a few hours.

Fig. 1.—Section of skin of a rabbit treated with a metallic mercury cream. The metal can be seen within the stratum disjunctum and in the cutis.

In general, extreme precaution should be taken to insure the freedom from mercury of all equipment. The microtome knife in particular, should be inspected frequently for adherent mercury. Dusts are sometimes troublesome, unless sections are kept covered whenever practicable. It is more conservative practice to withhold a positive identification of mercury unless at least some of the spheres can be seen within the substance of the section, or unless microchemical proof is undertaken.

TYPICAL FINDINGS

Metallic Mercury.—In our experiments, metallic mercury seldom occurred free in the body tissues. We have confirmed Zwick's observation that it may be seen in the subcutis following percutaneous inunction.[2] As he noted, the globules gradually lose their gloss and become roughened as though in process of chemical change. In in vitro experiments we noticed that a similar phenomenon takes place. When mercury is placed in physiologic saline, the droplets gradually lose their shininess. A grayish film appears. Calomel forms and the solution becomes alkaline. It appears probable that the tendency of mercury to react with components of the circulating fluids is responsible for the apparent failure to appear in the organs in metallic form.

We have further confirmed Zwick's finding that mercury globules are easily displaced during sectioning. We know of no certain remedy for this, but we repeat the suggestion that thick sections (15 microns) be used. Zwick thought to minimize the error, or at least to have it operate in his favor, by sectioning from within out. To this may be added the precaution of lowering the section on the reverse stroke of the microtome, so that the globules are not displaced backwards by the blade.

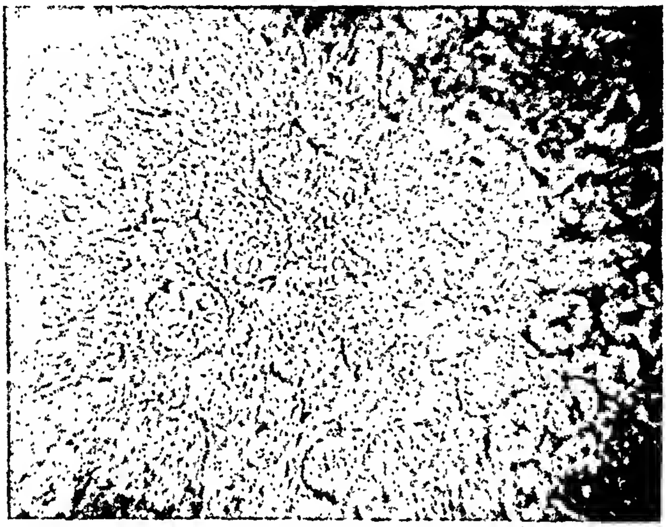


Fig. 2.—Section of kidney of a mouse injected intravenously with mercurous acetate showing mercury within the tubules.

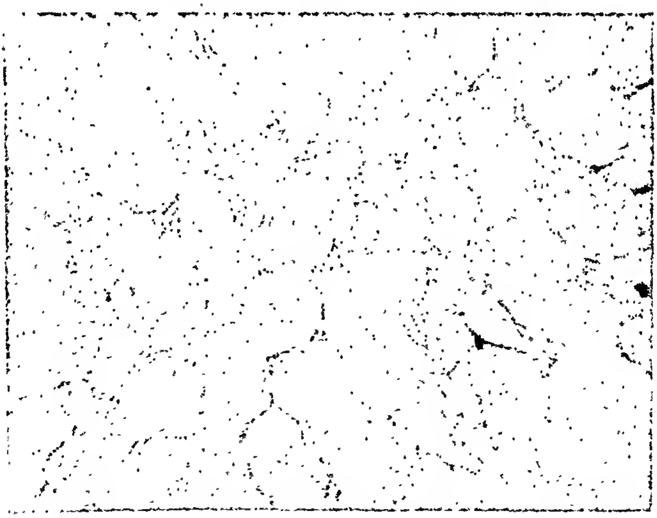


Fig. 3.—Section of kidney of a mouse injected intravenously with mercuric acetate showing mercury within the tubules and interstitially.

Fig. 1 shows the appearance and distribution of metallic mercury in the skin twenty-four hours after innuction from a vanishing cream vehicle.[9]

Mercurous Mercury.—Within a few minutes after the intravenous injection of mercurous acetate in laboratory animals (rats, rabbits, guinea pigs), both

mercurous mercury and mercuric mercury are found in the parenchymal cells and endothelial cells of the kidney. The latter form is preponderant. Even plain whole blood appears to have the capacity to oxidize mercurous salts to the mercuric form in vitro with great rapidity. We do not know what albuminates or complexes may form in the process, but the evidence that oxidation has taken place is based on the specificity of the thioglycollic acid reaction. It may be that subsequent metabolic or excretory processes ultimately lead to a reduction of valency, but the first effect is certainly one of oxidation. Fig. 2 shows mercurous mercury in the kidney of a mouse which had been poisoned by mercurous acetate.

Mercuric Mercury.—It was expected from the preceding observations that the divalent derivatives of this metal would appear in the liver and kidney as mercuric mercury. This proved to be the case. Systematic histochemical examinations have not been carried on over a long enough period of time to permit comment as to possible ultimate changes in valency of mercuric mercury in the body. Such a study might be of value in elucidating the questions of toxicity and excretion.

Fig. 3 shows the characteristic distribution of mercuric mercury in a mouse killed with mercuric acetate.

SUMMARY

Histochemical methods for the determination of mercury have been reviewed and some modifications suggested.

The use of thioglycollic acid for the histochemical differentiation of mercurous and mercuric mercury has been suggested and a method of procedure has been described.

We should like to thank Dr. S. W. Lee, Mr. Thomas Maren, Dr. Franz Herrmann, and Dr. Marion B. Sulzberger for constructive criticism and comment.

REFERENCES

1. Christeller, Erwin, and Sammartino, Rodolfo: Ueber den histochemischen Nachweis des Quecksilbers in den Organen, Ztschr. f. d. ges. exper. Med. 60: 11, 1928.
2. Feigl, Fritz: Qualitative Analysis by Spot Tests. New York, 1939, Nordeman Pub. Co., pp. 22-23.
3a. Justus, J.: Die Aktion des Quecksilbers auf das syphilistiche Gewebe. 2 Mitteilung, Arch f. Dermat. u. Syph. 60: 465, 1905.
3b. Justus, J.: Ueber Quecksilbervergiftung, Arch. f. Dermat. u. Syph. 75: 203, 1905.
4. Almkvist, Johann: Experimentelle Studien über die Lokalisation des Quecksilbers bei Quecksilbervergiftung, Nord. med. Ark. 2: 1. 1903.
5. Lombardo, C.: La micro- ed isto-chimica nella ricerca tossilogica del mercurio. Arch. di farmacol. sper. 7: 8, 1908.
6. Zwick, Karl G.: A Microscopic Study of Mercury Absorption From the Skin. J. A. M. A. 83: 1821, 1924.
7. Emich, Friedrich: Microchemical Laboratory Manual. New York, 1932, John Wiley & Sons, p. 86.
8. Claesson, P.: Ueber Thioglycolsäure. Ann. 187: 118. 1877.
9. Maren, T. H., and Edwards, B. B.: Studies in the Pharmacology of Mercury. I. Water Soluble Dispersion of Mercury, J. Am. Pharm. A. (Scient. Ed.) 32: 255, 1943.

LABORATORY METHODS

GENERAL

A SIMPLE MICROMETRIC APPARATUS FOR DETERMINING THE SEDIMENTATION RATE OF THE ERYTHROCYTES*

JULIAN L. ROGATZ, M.D., NEW YORK, N. Y.

SINCE Fahraeus first noted the significance of erythrocytic sedimentation in blood plasma, in 1918, this apparently simple phenomenon has been studied with increasing interest. Its usefulness in determining the presence or absence of infection in the body has been amply verified. Our present understanding of the erythrocytic sedimentation rate, its mechanism, behavior and significance in various diseases, has been admirably summarized by Cutler.[1]

Though the test has been generally accepted and is in constant daily use, there is no agreement as to a suitable apparatus for measuring the rate of settling of the red cells. Unwieldy arrangements with long tubes of wide bore, requiring comparatively large amounts of blood drawn from a vein, have given way to simpler and more practical devices utilizing tubes, or even pipettes, with much narrower bores, requiring not more than a drop of blood easily drawn from the tip of a finger; this is a great advantage in pediatric practice, permitting serial tests with little or no complaint from young patients.

There have been many of these micromethods, which have proved reasonably accurate. The technique usually requires the mixing of blood and anticoagulant separately before placing into the sedimentation pipette. In 1938 I compared what seemed the simplest of these methods, that described by Smith in 1936,[2] with the highly accurate Cutler macromethod and found it equally accurate.[3] Smith feels that in tubes of narrower bore than 2.5 mm. capillarity with its inherent drawbacks cannot be avoided. Thus his remains the only tube available in which capillarity does not occur and in which the figures can be compared directly with a standard venipuncture method. Though the merit of Smith's method may not be denied and its technique is easily mastered, it presents certain objections which militate against its general use by the practitioner and which must be eliminated in the ideal apparatus.

I referred at that time to the Landau modification of the early Linzenmeier-Raunert technique,[4] which seemed to fulfill most of the requirements of an ideal test, i.e., the drawing and mixing of blood and anticoagulant, and the sedimentation of the cells took place in one pipette, thereby eliminating all unnecessary,

*From the Abraham Jacobi Division for Children of the Lenox Hill Hospital, Service of Dr. Jerome S. Leopold.

Received for publication, May 30, 1943.

intervening steps. Careful sealing of the tip of the pipette to prevent loss of the contained specimen in the vertical position seemed a problem. A capillary pipette, similar to that used for the counting of white cells, was used. The column of blood was controlled by a "check syringe" device, which was fitted over the pipette and removed before setting into the rack. The gadget seemed complicated and was not available in this country.

During the past year, however, the Landau apparatus was manufactured locally,* and the "check syringe," on which the usefulness of the method depends, was simplified into an "aspirator" consisting of a metal screw and collar, which fits into a small piece of thick rubber tubing, the whole device being no more than two inches long and one-half inch wide.

The utter simplicity of this apparatus, which consists of only three parts, a pipette, an aspirator, and a rack, and permits of no further simplification of the sedimentation rate technique, impelled me to study its accuracy. I decided to use the proved Smith-Cutler tube as a control. Cases were taken at random as they entered the wards, usually older children because of the cooperation necessary for the drawing of the somewhat larger amounts of blood required for both tests, performed simultaneously. The results of the study on 100 cases, comprising 200 tests and deemed adequate, are presented in the accompanying table (see Table I).

APPARATUS

The Landau microsedimentation apparatus (see Fig. 1) consists of:

1. A capillary pipette 12.5 cm. long with a capillary bore 1 mm. in diameter, expanded into a bulb or mixing chamber 3 cm. from the upper end. Markings are etched at 12.5 mm. and 62.5 mm. from the lower tip, providing for a column of blood 50 mm. long and 1 mm. wide. The intervening space is graduated in millimeters.

2. An "aspirator" consisting of a metal screw with a milled head and its collar, 3.5 cm. long, which fits snugly into a piece of thick rubber tubing 4.5 cm. long. When screwed down, the entire length of the aspirator is 5.5 cm. Turning the screw in either direction controls the position of the column of blood in the pipette, over which it is fastened. When the screw is stationary, the column remains fixed.

3. A metal rack of simple construction with a rubber-cushioned floor and an adjustable rubber-cushioned cap. A hidden spring device in the floor permits depression of the base and holds the pipette vertically and hermetically sealed.

Control apparatus: the Smith micromethod described in a previous study.[2]

PROCEDURE

The upper end of the Landau pipette is fit into the rubber end of the aspirator, which is screwed tightly down. Holding the pipette in the palm of the hand, daggerlike (see Fig. 2), so that the thumb and the index finger control the screw, 5 per cent sodium citrate is drawn up into the pipette by revolving the screw, to the 12 mm. mark. A droplet of blood is then drawn from

*By the Clay-Adams Co., Inc., 44 East 23rd Street, New York City.

the pricked finger, as in a blood count, until the column reaches the 62.5 mm. mark, avoiding air bubbles by keeping the tip of the pipette in constant contact with the blood as it is expressed. Wipe the tip of the pipette clean to prevent later clotting of the blood at this point. The blood and citrate are thoroughly mixed by drawing the column into the ampula or chamber, stopping the lower meniscus a few millimeters below the opening of the chamber to avoid air bubbles. The column of blood is alternately depressed and raised, thus emptying and filling the chamber about seven times to insure proper mixing. The column is finally screwed down until the upper meniscus reaches the mark just below the mixing chamber. The lower tip of the pipette is pressed into the rubber floor of the rack while disengaging the aspirator. The upper end of the pipette is placed beneath the cap of the rack, where it will be held snugly and vertically. After standing for one hour, the millimeter level to which the red cells have fallen is read. The drawing and mixing of blood prior to the beginning of sedimentation should not take more than thirty seconds. The pipette should be cleaned with alcohol and ether after use.

The normal figures reported by Landau were 1 to 6 mm. for men and children under two years; 1 to 9 mm. for women and children over two years; 10 to 15 mm. were considered slightly increased values; 16 to 30 mm. moderately increased; over 30 mm. high. In general I think it sufficiently accurate to consider values under 10 mm. at the end of one hour normal and those up to 15 mm. slightly increased or "high normal" beyond which the rate has increasing significance.

COMMENT

Rapid sedimentation is dependent on the formation of red cells into large clumps or rouleaux, induced by alterations in the plasma, mainly an increase in fibrinogen, which results from the accumulation in the blood stream of certain products of tissue destruction during infections, certain intoxications, malignancy, etc. Cutler thought this change in the plasma proteins plays its predominant role in rouleaux formation, probably through surface dehydration or change in water balance on the surface of the red cells. Globulin, albumin, cholesterol, and electrolytes are involved to a lesser extent.

Smith contended that the influence of capillary exerted within the lumen of the narrow tube and the tendency to clot formation were inherent drawbacks in the pipette methods, but Morrison[5] indicated that the length of the tube used does not affect rouleaux formation, nor does the diameter as long as the tube remains perpendicular. Acceleration of sedimentation in tubes deviating from the perpendicular is dependent on the relative increase in vertical pathways or columns of falling cells. The smaller the diameter of the tube, the greater will be the relative increase in vertical pathways. Though Smith feels the capillarity present in tubes of less than 2.5 mm. bore a disadvantage, this has not proved sufficient in practice to materially affect the accuracy of the pipette method. Clotting of blood in pipettes happened but twice in this series and is therefore not a major objection.

The results obtained in this study correspond satisfactorily in all but four cases, those numbered 37, 42, 75, and 81. In each of these the rate in the Landau

pipette was well within normal limits while the Smith reading was slightly increased. Thus, in Case 37, there was a tonsillectomy in an apparently well child (the Smith reading was 15 mm. at the end of one hour while the Landau reading was 7 mm.). In Case 75, that of a child with purulent otitis media, the

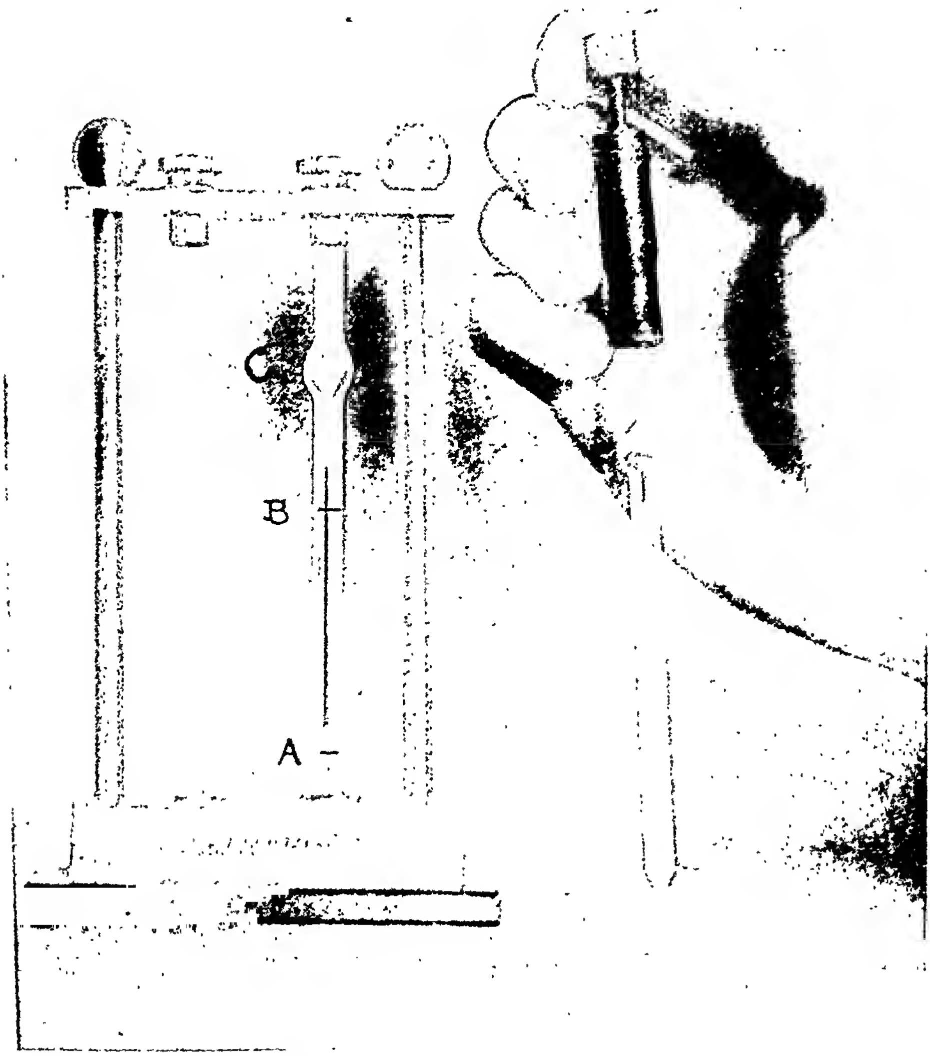


Fig. 1.—Showing Landau micrometric apparatus for sedimentation of the erythrocytes, consisting of metal rack for two pipettes, with one pipette in place, and the rubber aspirator, attached to the other pipette, held in hand. Sodium citrate 5 per cent is drawn to the 12.5 mm. mark (*A*) and blood is then drawn up to the 62.5 mm. mark (*B*), after which the column is drawn into the mixing chamber (*C*). Note specimen of blood in chamber of the pipette with aspirator and note that blood is not drawn completely into the chamber, thus avoiding air bubbles. The pipette in the rack contains a sedimenting column of blood.

Smith reading of 15 mm. seemed more related to the clinical state than the corresponding Landau value of 4 mm. The most marked discrepancy occurred in Patient 81, recovering from an acute tonsillitis, whose rate according to the Smith method was 17 mm. and only 7 mm. by the Landau method.

In the remaining 96 cases the figures corresponded favorably. In some, the high values were somewhat higher in the Landau pipette, while in others

Fig. 2.—Showing method of holding pipette with aspirator attached as blood is withdrawn from tip of a finger.

this was true of the Smith-Cutler tube. The variations, as high as 18 mm. in one case, averaged 7 mm. in 33 patients in whom the higher value appeared in the Landau tube. In 18 cases the situation was reversed, the higher values appear-

TABLE I

TABLE OF CASES

CASE NUMBER	AGE YEARS	DATE	DIAGNOSIS	SMITH MICROMETRIC MM. ½ HR.	SMITH MICROMETRIC MM. 1 HR.	NEW LANDAU MICROMETRIC MM. ½ HR.	NEW LANDAU MICROMETRIC MM. 1 HR.
1	10	9/ 1/42	Tonsillectomy	1	3	2 sharp	4 sharp
2	8	9/ 1/42	Tonsillectomy	1	2	2 sharp	3 sharp
3	9	9/ 4/42	Convalescent bronchitis	8	16	2 blurred	25 sharp
4	7	9/ 4/42	Convalescent "grip"	13	20	? blurred	38 sharp
5	11	9/ 5/42	Acute sinusitis	18	24	10 sharp	25 sharp
6	10	9/ 5/42	Convalescent pneumonia	6	16	2 blurred	26 sharp
7	11	9/13/42	Melanoma ? of eye	4	9	1 blurred	4 sharp
8	8	9/13/42	Post tonsillectomy	10	18	2 blurred	28 sharp
9	9	9/14/42	Accessory scaphoids	2	4	1 sharp	2 sharp
10	8	9/15/42	Cleft palate	2	6	2 sharp	3 sharp
11	8	9/15/42	Convalesc. rheumatic fever	1	5	1 sharp	6 sharp
12	11	9/16/42	Convalescent bronchitis	5	12	2 blurred	5 blurred
13	6	9/16/42	Convalescent pneumonia	9	16	1 blurred	20 blurred
14	4	9/17/42	Acute appendicitis ?	7	18	10 blurred	17 sharp
15	8	9/17/42	Convalesc. rheumatic fever	2	6	1 sharp	3 sharp
16	5	9/24/42	Catarrhal otitis media	24	29	1 blurred	41 sharp
17	10	9/28/42	Convalescent "grip"	4	8	1 sharp	3 sharp
18	9	9/28/42	Asthmatic bronchitis	7	17	2 sharp	20 sharp
19	9	9/29/42	"Grip"	5	23	20 sharp	28 sharp
20	6	9/29/42	Convalescent bronchopneumonia	8	17	1 sharp	23 sharp
21	8	10/ 6/42	Convalescent "grip"	6	18	2 blurred	26 sharp
22	6	10/ 9/42	Acute catarrhal jaundice	8	16	2 sharp	29 sharp
23	9	10/ 9/42	Lobar pneumonia	13	18	8 sharp	22 sharp
24	9	10/15/42	Convalescent bronchopneumonia	8	15	7 blurred	23 sharp
25	7	10/15/42	Aphthous stomatitis	7	14	2 sharp	10 sharp
26	9	10/23/42	Tonsillectomy	2	5	2 sharp	4 sharp
27	6	10/23/42	Convalescent "grip"	6	12	4 sharp	12 sharp
28	5	10/23/42	No pathology apparent	6	12	4 sharp	12 sharp
29	9	10/23/42	Acute peritonitis	13	23	13 blurred	22 sharp
30	8	10/28/42	No pathology apparent	14	18	10 blurred	24 sharp
31	10	10/28/42	Convalescent "grip"	5	10	3 sharp	5 sharp
32	14	11/ 4/42	No pathology apparent	2	5	3 sharp	6 sharp
33	4	11/ 4/42	Convalescent "grip"	4	9	3 sharp	6 sharp
34	10	11/ 5/42	"Grip" ?	4	12	2 blurred	15 sharp
35	8	11/ 5/42	Convalescent "grip"	12	20	12 blurred	23 sharp
36	9	11/ 6/42	Appendicitis ?	8	15	2 blurred	13 blurred
37	6	11/ 6/42	Tonsillectomy	8	15	2 sharp	7 blurred
38	11	11/ 6/42	Pneumonia	21	28	6 blurred	20 blurred
39	7	11/10/42	"Grip"	10	18	2 sharp	13 blurred
40	6	11/10/42	Acute bronchitis	6	12	3 sharp	9 sharp
41	8	11/13/42	Tonsillectomy	4	10	3 sharp	4 sharp
42	1	11/13/42	Pertussis	12	21	6 blurred	10 blurred
43	10	11/18/42	Tuberculous hip ?	4	8	2 sharp	4 sharp
44	7	11/18/42	"Grip"	5	12	2 sharp	11 blurred
45	5	11/19/42	Convalescent pneumonia	13	20	3 blurred	13 blurred
46	4½	11/19/42	Gastroenteritis	5	10	2 blurred	5 sharp
47	12	11/27/42	Virus (atypical) pneumonia	5	10	4 sharp	9 sharp
48	5	11/27/42	No pathology apparent	3	10	1 sharp	3 sharp
49	8	12/ 2/42	Acute bronchopneumonia	22	27	5 blurred	20 blurred
50	9	12/ 3/42	Fracture of humerus	2	8	2 sharp	3 sharp
51	8	12/ 3/42	Acute bronchopneumonia	20	23	19 sharp	21 sharp
52	5	12/ 3/42	Acute mastoiditis	34	35	27 sharp	30 sharp
53	7	12/ 3/42	Inguinal hernia	8	18	1 sharp	2 sharp
54	8	12/ 5/42	T.B. cervical adenitis	5	10	1 sharp	2 sharp
55	12	12/ 5/42	Convalesc. appendectomy	10	18	1 sharp	4 sharp
56	8	12/ 7/42	Upper respiratory infection	14	22	2 blurred	28 sharp
57	9	12/ 7/42	Hemangioma	6	12	15 blurred	33 sharp
58	12	12/ 8/42	Convalesc. appendectomy	3	8	1 sharp	2 sharp

TABLE I—CONT'D

CASE NUMBER	AGE YEARS	DATE	DIAGNOSIS	SMITH MICROMETRIC MM. ½ HR.	SMITH MICROMETRIC MM. 1 HR.	NEW LANDAU MICROMETRIC MM. ½ HR.	NEW LANDAU MICROMETRIC MM. 1 HR.
59	5	12/ 8/42	Chronic mastoiditis	5	17	1 sharp	13 blurred
60	10	12/10/42	Acute tonsillitis	15	26	2 sharp	26 sharp
61	8	12/10/42	Convalescent pneumonia	20	25	12 sharp	24 sharp
62	8	12/15/42	Chronic cardiovascular disease	15	23	8 blurred	23 sharp
63	6	12/15/42	Convalesc. C.S. meningitis	2	9	1 sharp	10 sharp
64	6	12/21/42	Tuberculous hip	8	15	2 sharp	20 sharp
65	8	12/21/42	Tuberculous pleurisy	6	13	1 sharp	9 sharp
66	6	12/21/42	Convalescent meningitis	3	9	1 sharp	2 sharp
67	8	12/21/42	Chronic cardiovasc. disease	3	9	1 sharp	2 sharp
68	9	12/31/42	Convalescent pneumonia	26	28	39 sharp	42 sharp
69	6	12/31/42	Acute tonsillitis	17	22	10 blurred	20 sharp
70	8	12/31/42	Tuberculous pleurisy	2	8	1 sharp	7 sharp
71	7	12/31/42	Acute bronchopneumonia	7	24	1 sharp	36 sharp
72	6	1/ 7/43	Pleurisy	19	23	2 blurred	25 blurred
73	3	1/ 7/43	Eczema	19	24	15 blurred	24 sharp
74	2	1/ 8/43	Acute tonsillitis	27	29	21 sharp	28 sharp
75	6	1/ 8/43	Acute otities media purulenta	9	15	2 sharp	4 sharp
76	8	1/11/43	Wrist injury	4	7	4 sharp	5 sharp
77	8	1/11/43	Cervical adentitis (acute)	23	27	35 sharp	38 sharp
78	6	1/14/43	Acute tonsillitis	18	22	10 sharp	16 sharp
79	8	1/14/43	Acute catarrhal otitis media	25	28	32 sharp	38 sharp
80	8	1/15/43	Convalescent pleurisy	2	5	1 sharp	2 sharp
81	6	1/15/43	Convalescent acute tonsillitis	8	17	1 sharp	7 blurred
82	10	1/16/43	Acute mastoiditis	12	21	15 blurred	26 sharp
83	6	1/16/43	T.B. cervical adenitis	3	5	2 sharp	4 sharp
84	14	1/18/43	Tuberculous hip	22	30	15 sharp	18 sharp
85	10	1/18/43	Fracture of elbow	1	3	1 sharp	2 sharp
86	5	1/19/43	Chronic cardiovalv. disease	2	6	2 sharp	5 sharp
87	4	1/19/43	Acute tonsillitis	14	23	27 sharp	38 sharp
88	13	1/21/43	Chorea	2	4	2 sharp	3 sharp
89	6	1/21/43	Clubbed feet	9	17	11 blurred	25 sharp
90	6	1/25/43	"Grip"	2	4	1 sharp	2 sharp
91	12	1/25/43	Convalescent rheumatic fever	2	6	1 sharp	2 sharp
92	8	1/26/43	"Grip"	5	15	1 blurred	29 sharp
93	4½	1/26/43	"Grip"	4	14	2 sharp	13 blurred
94	4½	1/28/43	Acute ethmoiditis (acute hemolytic anemia)	31	35	41 sharp	44 sharp
95	2	1/28/43	Tonsillectomy	6	20	20 blurred	33 sharp
96	7	1/28/43	Convalescent meningitis	30	34	37 sharp	40 sharp
97	5	1/28/43	Convalescent appendicitis	2	8	1 sharp	11 sharp
98	14	1/28/43	Tuberculosis of Spine	14	27	26 sharp	39 sharp
99	8	1/28/43	"Grip"	3	8	1 sharp	3 sharp
100	13	2/12/43	Hypertension	7	14	6 sharp	12 sharp

ing in the Smith-Cutler tube, the greatest variation being 12 mm., the average 5 mm. These differences are not great when it is considered that they occurred in sedimentation rates which were very rapid. They are, therefore, not significant.

After reading a number of sedimentation tests, it soon becomes apparent that there may be two types of reaction. The separation of red cells from plasma is usually indicated by a sharp demarcation. Frequently, however, it may be diffuse or blurred. This happens occasionally in other tubes with larger bores, as it did a number of times in the Cutler tubes. It is most likely to occur early in

a test the sedimentation of which proves rapid. There is a gradual tapering of color from above downward, the pale plasma deepening into the pinkish color of sinking erythrocytes, which merges into the deep red of packed cells. By the end of an hour the blurring was usually replaced by a sharp line. In only nine cases of this series did blurring persist after one hour. In Case 36 it cleared in seventy minutes.

As the cells pack, the diffused area covers only a few millimeters and the line of probable demarcation can be easily estimated within one or two millimeters, which is sufficiently accurate for all practical purposes. If a reading is deemed unsatisfactory, it is possible to reattach the aspirator to the pipette, start drawing the column into the mixing chamber before releasing pressure on the floor of the rack, and allow the cells to sediment anew, provided the blood has not clotted in the pipette. The second reading may be more satisfactory.

SUMMARY

The new Landau microsedimentation apparatus is described, and its reliability is demonstrated in a series of 100 cases, in which the proved Smith-Cutler method was used as a control. The apparatus and technique have been reduced to their simplest possible form by successfully combining the several necessary steps, namely, the drawing and mixing of blood and anticoagulant and the sedimenting of the red cells, into one uninterrupted procedure accomplished in one pipette. Only three pieces of apparatus of the simplest possible construction are required: the pipette, an aspirator, and a rack. Not more than $\frac{1}{20}$ c.c. of blood is used, and the time required from puncture of the finger to the beginning of sedimentation is about thirty seconds. The set is so compact that it may be placed in a small corner of one's bag. No previous experience or unusual skill is necessary for the efficient use of this method. A test of acknowledged significance thus becomes universally available to practitioner and specialist alike, equally useful at the bedside or in the laboratory.

CONCLUSION

The new Landau erythrocytic microsedimentation test fulfills the requirements of the ideal method. It is deemed worthy of further trial. Its reliability having been adequately demonstrated, it is suggested that this technique and apparatus may help to establish the desired standardization of the sedimentation rate test.

REFERENCES

1. Cutler, J. W.: Present Concept of Erythrocyte Sedimentation Rate. New Internat. Clin. 3: 62, 1940.
2. Smith, C. H.: A Method for Determining the Sedimentation Rate and Red Cell Volume in Infants and Children With the Use of Capillary Blood, Am. J. M. Sc. 192: 73, 1936.
3. Rogatz, J. L.: Microsedimentation of the Erythrocytes in Infants and Children, Am. J. Dis. Child. 56: 1037, 1938.
4. Landau, A.: Microsedimentation. Am. J. Dis. Child. 45: 691, 1933.
5. Morrison, I.: Observations on Blood Sedimentation Mechanism. Am. J. Clin. Path. 11: 578, 1941.

PORTABLE APPARATUS FOR ESTIMATING THE PERIPHERAL BLOOD FLOW IN SHOCK*

HERBERT S. WELLS, M.D., AND JOHN J. THOMPSON, A.B., WINSTON-SALEM, N. C.

SKIN pallor in shock suggests that the peripheral blood flow is reduced, but it is difficult to estimate the degree of this reduction from the intensity of the pallor or other clinical signs. The one series of measurements carried out on human cases[1] indicates the usefulness of quantitative information on blood flow for gauging the degree of developing circulatory failure and the response to transfusion. The direct plethysmographic method, however, commonly used to measure blood flow, requires cumbersome equipment not easily adapted to studies at the bedside or in the operating room or casualty station. The method to be described, while not so accurate as the more elaborate equipment of the physiologic laboratory, is practical in that it gives consistent readings which serve well for comparative studies, and because the apparatus is portable and easy to operate.

The apparatus is essentially a small air plethysmograph consisting of a cuff, 1¼ inch wide, which supports a thin rubber bag against the skin. As the limb swells, following quick inflation of a centrally located sphygmomanometer cuff, air is expelled from the cuff bag, whence it passes to one end of a horizontal pipette (1 c.c. calibrated in 0.1 c.c. units) containing a droplet of water, the movement of which measures the rate of swelling of the limb. The distal end of the pipette connects to a second rubber bag upon which a lead weight floats. This constant pressure or "reservoir" bag (*R B* in Fig. 1) maintains a nearly uniform pressure of 6 cm. water in the whole system, which is sufficient to inflate the cuff for contact with the skin. Movement of the water drop is timed by counting swings of a pendulum whose period is 0.5 second. The number of pendulum swings for successive 0.1 c.c. excursions of the water drop are recorded. These data, together with the volume of the limb enclosed by the cuff, which is indicated by markings on the cuff, enable calculation of the blood flow in c.c. per 100 c.c. of limb per minute. An alignment chart is used, in practice, to facilitate the calculations.

It has been found that the cuff bag need not completely surround the limb. A bag 7 inches long, made from ¾ inch Penrose drainage tubing and cemented to a cuff 15 inches in length, may be applied to a limb segment of volume up to 250 c.c. For use on the leg a longer cuff and bag may be required.

Several forms of the apparatus have been designed, but the most compact and readily portable type, shown in the upper sketch of Fig. 1, has proved most practical. All necessary equipment except the sphygmomanometer (aneroid

*From the Laboratories of Physiology and Pharmacology, Bowman Gray School of Medicine, Winston-Salem, N. C.

Aided by a grant from the Council on Physical Therapy of the American Medical Association.

Received for publication, April 27, 1943.

type) is contained in a box which is $4\frac{1}{4}$ x 9 x $1\frac{1}{4}$ inches when closed. The traps, *T* in the diagram, which prevent loss of the water drop, enable the box to be carried in any position. The pipette is mounted on a strip of wood, which may be turned forward, allowing the water traps to lie in a horizontal position so the cover may be closed. The cuff, the metal paper clip (not shown) used for holding the cuff in place on the limb, and a tourniquet for the wrist (18 inches of 1 inch Penrose tubing) may be placed in the box when not in use. A short thermometer, suitable for measurement of the skin temperature, is held in the upper part of the metal clip shown inside the cover of the box. The lower part of this clip serves to clamp the pendulum when not in use.

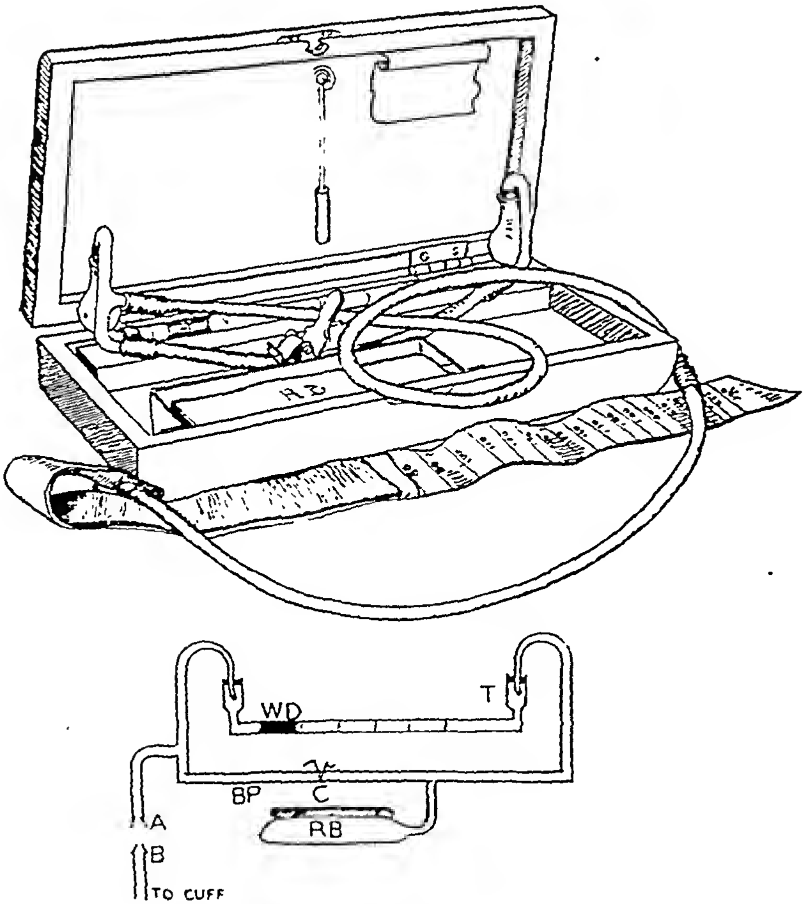


Fig. 1.—Drawing and diagram of blood flow apparatus. *W D*, water drop in 1 c.c. pipette; *T*, water traps at each end of the pipette; *A* and *B*, metal connectors on tubing to the cuff bag; *B P*, by-pass tube, which is opened or closed by clamp, *C*; *R B*, reservoir bag, 4 inches long, similar to the cuff bag, and having a lead weight of 150 Gm. "floated" upon it.

A by-pass, *B P*, which is closed by the clamp, *C*, during the determination, is opened to allow air to pass between the cuff bag and the reservoir bag during inflation of the bag system or during movements of the patient which might otherwise cause the water drop to be blown out of the pipette. To inflate the bag, the cuff is first fitted snugly, but not tightly, to a segment of the arm or leg,

which should be as nearly circular in cross section as possible. The metal tubing connectors, *A* and *B*, are separated. The operator blows into *A* until the reservoir bag, *R B*, is inflated. Pinching off the rubber tube above *A*, he then connects *A* and *B*. When the tube is released, air from *R B* passes back into the cuff bag.

Spontaneous variations of blood flow are reduced to a minimum, if the circulation to the hand is occluded by a tight tourniquet of Penrose tubing wrapped tightly just above the wrist.

The accuracy of the method was tested by comparison of simultaneous recordings, which measured the swelling rates following venous occlusion of (a) the whole forearm inserted in a water-jacketed air plethysmograph and (b) of a segment of this forearm, encircled by the cuff plethysmograph. For recording purposes a slightly modified form of the apparatus was used in which the reservoir bag system was replaced by a constant pressure water reservoir, from the open end of which air was displaced at atmospheric pressure to a volume recorder. A second recorder registered the displacement of air from the larger plethysmograph. The recorders consisted of soap films formed across the ends of horizontal glass tubes. Images of the bubbles, which increased in size and approached each other as air entered from the respective plethysmographs, were projected at convenient magnification across the horizontal slit of a 12 cm. recording camera. Time in seconds, calibration lines for the two recorders, and a mercury manometer record of the pressure applied to the collecting cuff were also photographed. Soap film recorders (first used in this laboratory) respond to pressure changes of less than 0.005 mm. H_2O; and errors of amplitude, time lag, and maximum rate of response during tests, with simple harmonic volume changes, are imperceptible up to a frequency of 3 to 5 cycles per second. The natural frequency of the larger recorder was 12 to 15 per second.

The cuff plethysmograph, as tested in this manner on 5 normal subjects (23 determinations) gives values of the blood flow, per unit volume of limb, which are regularly low. The mean error is -28 ± 11.3 per cent, the range being from -10 to -47 per cent.

It is evident that the absolute values obtained cannot be relied upon to give better than an estimate of the actual blood flow. The readings, however, are consistent and reproducible, and comparative measurements are reliable. Furthermore, the variability (standard deviation as per cent of the mean) of readings on normal subjects is not greater than is the case for more accurate methods. The mean of measurements on 54 normal subjects was 2.65 ± 0.96 c.c. per 100 c.c. per minute. All subjects were comfortably warm, at room temperatures between 21° C. and 30° C. and with forearm skin temperatures from 29° C. to 34° C.

In most cases it makes no difference whether the cuff is placed around the upper, middle, or lower third of the forearm; the same value for blood flow is obtained in each case. This indicates a remarkable uniformity (from which, however, some subjects show considerable departures) of the swelling of all portions of the limb. It seems evident that the chief cause of the error of the cuff plethysmograph is referable to the slight compression of blood vessels lying be-

neath it rather than to the location of the cuff on a portion of the arm which swells at less than the average rate for the limb as a whole.

During the past year the method has been tried in clinical practice in several hospitals on over a hundred patients, including a number suffering from various forms of circulatory failure (cardiogenic, vasogenic, hematogenic types). The cases, briefly reported in Table I, may suffice to indicate the low levels of peripheral blood flow found in hematogenic shock. The increase of the flow to or toward normal levels, following transfusion, is noteworthy. The tendency of the blood flow to vary inversely with the hematocrit readings in Case 4, is of particular interest, since a blood flow determination may be made in a fraction of the time required for a hematocrit. In general, we may say that a blood flow value of less than 1.0 c.c. per 100 c.c. per minute, which persists after pain, nausea, cold, or other confusing factors have been eliminated, is highly suggestive of peripheral circulatory failure of the hematogenic type. Further clinical studies will of course be necessary to determine the range of usefulness of the method.

TABLE I

CASE NO.	TIME AFTER INJURY	BLOOD PRESSURE	HEMATOCRIT	BLOOD FLOW	REMARKS
	hours	mm. Hg	per cent cells	c.c. per 100 c.c. per minute	
1	5	96/60	-	0.8	Gastroenterostomy followed by profuse hematemesis, seven hours. Transfusions, whole blood, 1500 c.c. to tenth hour; 1000 c.c. twenty-first hour.
	10	70/50	-	0.4	
	21	120/76	-	2.4	
2	1	50/?	-	0.5	Stab wound of thorax. Transfusion 500 c.c. whole blood first to fifth hours. Bleeding not controlled until third hour.
	3	68/?	-	0.6	
	5	110/74	-	1.1	
3	4	50/?	-	0.7	Post-partum hemorrhage. Transfusion, 800 c.c. glucose, 500 c.c. plasma from fourth to sixth hours.
	5	108/80	-	2.7	
	6	120/82	-	3.6	
4	0.5	-	38.2	-	Severe second degree burn. Received 5300 c.c. plasma during thirty hours.
	6	-	45.2	2.3	
	17	-	58.8	0.6	
	108	-	38.7	2.5	

The blood flow apparatus serves a second useful function in shock cases, for it enables one to determine the systolic blood pressure in those patients (unexpectedly numerous, in our experience) in which the usual methods fail and the pressure is reported as "unobtainable." As the sphygmomanometer cuff pressure is lowered to the systolic level, blood entering the forearm, even if its weak pulsations cannot be heard with the stethoscope or palpated at the wrist, will cause the water drop in the pipette of the blood flow apparatus to move. A very sharp measure of systolic pressure can always be determined in this way.

REFERENCE

1. Freeman, N. E., Shaw, J. L., and Snyder, J. C.: The Peripheral Blood Flow in Surgical Shock. The Reduction in Circulation Through the Hand Resulting from Pain, Fear, Cold, and Asphyxia, With Quantitative Measurements of the Volume Flow of the Blood in Clinical Cases of Surgical Shock, J. Clin. Investigation 15: 651, 1935.

A RESPIRATORY CHAMBER FOR CHRONIC EXPOSURE TO GASES*

A. T. MILLER, JR., CHAPEL HILL, N. C.

THE respiratory chamber described in this paper is a modification of the one used by the author in previously reported work (Miller, 1940[1]) on the effects of chronic exposure to carbon dioxide. The apparatus itself has not previously been described. The chamber now in use in this department was designed specifically for studies on chronic carbon monoxide exposure, but is readily adaptable to the study of other toxic gases, including war gases.

The over-all dimensions of the chamber are 11 feet by 7 feet by 6 feet. The actual gas chamber is 8 feet by 7 feet by 6 feet, giving a capacity of 336 cubic feet. The remainder of the capacity is accounted for by the space between the double entrance doors. The chamber will readily accommodate three human subjects, or 12 dogs, or 200 rats.

The chamber is constructed of 24 gauge sheet metal placed over a framework of two by fours. The metal is fastened to the framework with galvanized nails so that the junction between nail heads and sheet metal can be soldered. All seams are soldered and the entire chamber is painted inside and out with heavy floor enamel. Control experiments indicate that a gas mixture placed in the chamber suffers no measureable change in composition during forty-eight hours.

The chamber has a wooden floor elevated four inches from the sheet metal bottom and sealed to prevent trapping of gases between the two. The entrance doors are built of 1½ inch redwood, reinforced by 1 inch angle iron to prevent warping. The door overlaps the door facing 2 inches on all sides, and when the door is closed, it is sealed by a gasket consisting of rubber tubing set in a continuous groove on the inner surface of the door overlap. Each door is closed by two commercial cold room handles which exert enough pressure to collapse the rubber tubing gaskets against the door facing. The double door entrance minimizes changes in gas composition produced by entering and leaving the chamber during the course of an experiment.

Two windows, each 3 feet by 2 feet, are placed in the chamber for illumination and observation. Additional illumination is provided by an overhead light bulb, and an even mixture of gases is maintained by an oscillating fan. Electric wires are led into the chamber through porcelain insulators and are sealed with cement.

A continuous circulation of air through the chamber maintains a normal pressure of oxygen and removes carbon dioxide. As shown in Fig. 1, this circulation is provided by an 8 inch electric fan mounted directly into a funnel-like expansion of the 4 inch tube leading into the chamber. A 4 inch exit tube leads

*From the Department of Physiology, School of Medicine, University of North Carolina.
Aided by a grant-in-aid from the Sigma Xi Alumni Research Fund.
Received for publication, May 5, 1943.

to the outside of the building. The gas to be studied is led into the ventilating tube through a side tube as shown in the diagram.

The maintenance of a desired gas concentration in the chamber requires two things: a constant rate of ventilation and a constant rate of flow of the gas into the ventilating tube. Flowmeter tests indicate that an electric fan kept in good working condition maintains a very constant ventilation rate. A constant inflow of gas requires a source of gas under a constant pressure, with adjustment of the rate of flow by suitable reducing valves. Illuminating gas is a convenient source of carbon monoxide in amounts large enough for continuous flow. The low pressure at which this gas is ordinarily delivered and the fluctuations in rate of flow require a system for storing the gas under pressure for delivery to the gas chamber. The details of this system are shown in Fig. 1. The gas flows from the jet into a 50 liter Tissot spirometer. This serves as a reservoir from which gas is compressed into a pressure tank. The compression pump is a Crowell rotary number OD with a maximum pressure rating of 25 pounds and a free air flow of 2 cubic feet per minute. The pump is operated by a ¼ H. P. motor. The pressure tank is equipped with an automatic pressure regulator ("Pressuretrol" type L 404-2, made by the Minneapolis-Honeywell Regulator Company) with a pressure range of 2 to 50 pounds. This pressure regulator is connected to a pump motor through a Colt "Noark" automatic switch. The pressure regulator is set for a cutin pressure of 18 pounds and a differential of 2 pounds, so that gas is always delivered from the pressure tank to the respiration chamber at a pressure between 18 and 20 pounds. The outlet from the pressure storage tank is provided with a gate valve for cutting off the flow completely at the end of an experiment and with a Hoke ammonia control valve for fine adjustment of flow. This valve permits accurate control from full flow down to 5 bubbles per minute. In practice, the concentration of carbon monoxide in the respiration chamber can be controlled within 0.001 per cent during a 6 hour exposure period.

The absolute variation in rate of flow of gas into the tank may be determined as follows. Gas is delivered from the storage tank at a pressure which steadily declines from 20 to 18 pounds over a period of about ten minutes. At this point, the pump cuts on and the pressure is built up to 20 pounds again in about one and one-half minutes. The average delivery pressure then is 19 pounds with a range of ± 1 pound, or 5 per cent of the total pressure. This means that a given rate of flow can be maintained within the limits of ± 5 per cent, and this is proved by the fact that the fluctuations in the flowmeter reading are uniformly within these limits (see Fig. 2). If a still more uniform rate of flow is necessary, it can be obtained either by using a pressure regulator with a smaller differential range or by inserting a constant flow valve (such as those used on oxygen cylinders) between the pressure tank and the respiration chamber.

The operation of the entire gas flow assembly is made completely automatic by providing the bell of the Tissot spirometer with an automatic checking device. The rise of the bell is checked at such a height that the depth of the bell below the water level is greater than the gas pressure in the main, in centimeters of water. The pressure in the bell increases until it equals the main pressure and the flow of gas from the main ceases. Guy ropes attached to the top of the spirometer bell check it at the desired point.

The concentration of carbon monoxide in the respiration chamber may be determined by the usual analytic methods. A great saving of time and effort is made by the use of a carbon monoxide indicator (Mine Safety Appliances Company) which requires only about two minutes for a determination.

Routine determination of the carbon monoxide concentration in the chamber may be obviated in the following way. The flowmeter is calibrated against the carbon monoxide concentration in the chamber throughout the range of concentrations to be used. It should be emphasized that this calibration must be experimental, since the logarithmic relation between flowmeter pressure differential and rate of flow breaks down at rates of flow high enough to produce turbulence. Once the calibration is performed, the desired carbon monoxide concentration is obtained by adjusting the fine control valve to give the proper flowmeter reading. The oxygen and carbon dioxide concentrations should be checked occasionally, although they have never been found outside normal limits in work in this department. The relative humidity is recorded at the end of an exposure period by the wet bulb method. This may be done without entering the chamber by inserting the wet bulb thermometer through a rubber stopper into a flask through which a stream of air from the chamber is drawn by suction. The relative humidity has never been observed to rise above that of the outside atmosphere even with a full complement of experimental animals in the chamber.

If the effects of carbon monoxide are to be studied uninfluenced by other constituents of illuminating gas, these constituents must be removed. Most artificial gas is a mixture of coal gas and water gas. The Chapel Hill city gas has the following percentage composition:

Carbon monoxide	23.4
Hydrogen	36.8
Methane	17.8
Illuminants (unsaturated hydrocarbons)	8.6
Nitrogen	8.5
Carbon dioxide	3.7
Oxygen	1.2
Benzene (not included in analysis furnished by gas company but usually present to the extent of 1 or 2 per cent).	

This is a characteristic composition for manufactured gas, although the relative proportions of the constituents may vary in different communities. Of these, hydrogen, nitrogen, and methane are simple asphyxiants; that is, they are inert gases which act by excluding oxygen from the lungs when present in high concentration. The unsaturated hydrocarbons have anesthetic properties in high concentration (about 60 per cent), but are simple asphyxiants in low concentrations. All of these gases are present in extremely low concentration in the final mixture of air and gas delivered to the chamber (less than 0.2 per cent for the sum of all of them when the final carbon monoxide concentration is 0.05 per cent) so that their effect is negligible and they need not be removed. The final concentration of carbon dioxide under the same conditions is less than that of atmospheric air. Benzene is the only constituent aside from the carbon monoxide which is present in sufficiently high concentration to exert specific effects. The greater toxicity of illuminating gas compared with pure carbon

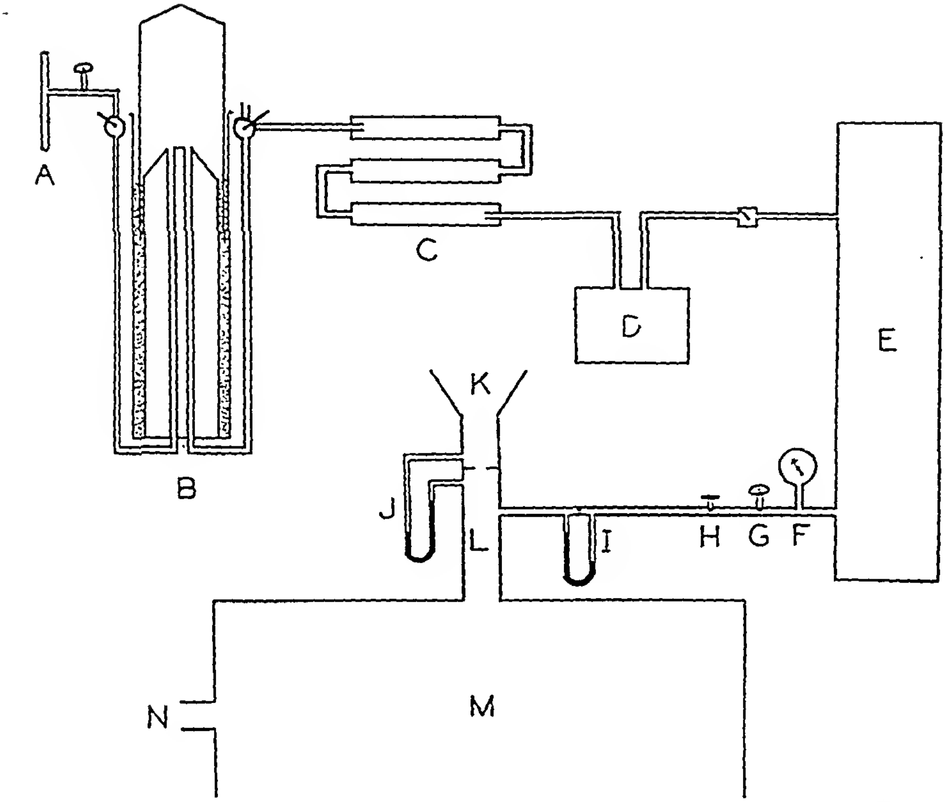


Fig. 1.—Diagram of assembly for controlling gas flow and ventilation. *A*, gas main; *B*, Tissot spirometer; *C*, charcoal absorption tubes; *D*, compression pump; *E*, pressure storage tank; *F*, pressure gauge; *G*, gate valve; *H*, reducing valve; *I* and *J*, flowmeters; *K*, position of ventilating fan; *L*, ventilation tube; *M*, gas chamber; *N*, exit tube of ventilating system. The motor which operates the pump and the automatic pressure control are not shown in the diagram.

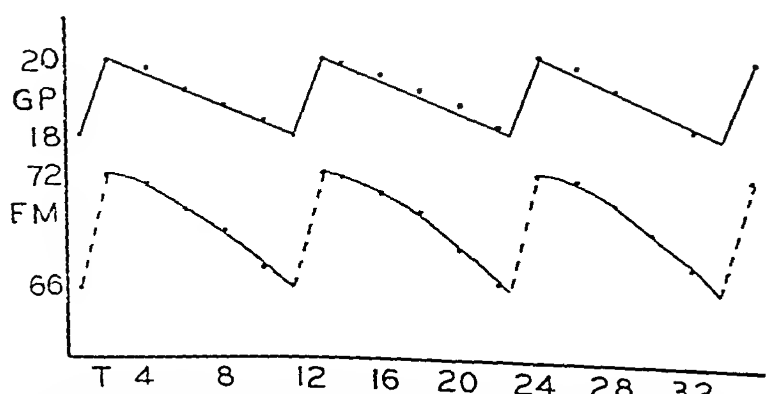


Fig. 2.—Relation between storage tank gauge pressure and rate of flow. Abscissa, time in minutes. Ordinate, *FM* = flowmeter readings in mm. glycerin, *GP* = gauge pressure in pounds.

monoxide is usually attributed to the presence of benzene. This may be removed quantitatively from the illuminating gas by passage through absorption tubes containing activated charcoal, as shown in Fig. 1. The completeness of removal may be tested by bubbling the gas through a test bottle containing a dilute solution of formaldehyde in sulfuric acid (5 c.c. of 40 per cent formaldehyde diluted to 100 c.c. with concentrated sulfuric acid). A reddish-purple color indicates the presence of benzene.

Only slight modifications are required to adapt the chamber to the use of other toxic gases, including war gases. If the gas to be used is lighter than air, no essential modification is necessary, since the gas leaving the exit tube of the chamber is quickly dissipated by dispersion. If heavier-than-air gases or vapors are to be used, the exit tube should lead through a suitable trap for absorption of the gas or vapor. If corrosive gases, such as chlorine, are used, all surfaces with which the gas comes in contact must be painted with one of the resistant paints now commercially available.

SUMMARY

A respiration chamber and gas train for chronic exposure to carbon monoxide are described. The assembly is automatic in operation and requires no attention during an eight-hour exposure period. The slight modifications necessary for adapting the chamber to the use of other toxic gases are described.

REFERENCE

1. Miller, A. T., Jr.: Acclimatization to Carbon Dioxide. A Study of Chemical and Cellular Changes in the Blood, Am. J. Physiol. 129: 524, 1940.

IMMUNOLOGIC REACTIONS FOLLOWING TYPHUS VACCINATION IN ARMY PERSONNEL*

JAMES H. PARK, M.D.† AND FREDERICK M. OFFENKRANTZ, M.D.‡

THE term typhus as generally used refers to three groups of rickettsial diseases: 1. Classical typhus (which includes Murine typhus) and a louse- or flea-borne group. 2. Rocky Mountain Spotted fever, which is caused by a tick-borne group. 3. Scrub typhus and tsutsugamushi fever, which are mite-borne diseases. The known role of typhus as a decimater of civilian and military populations in wartime is too well known to be commented upon further. The form of typhus which has been responsible for this stigma is the Classical group, epidemic throughout Europe. Because of its importance as a public health measure, the War Department, even prior to the present conflict, began immunization of all military personnel destined to enter potential or actual typhus areas. The form of vaccine employed was the rickettsial preparation grown by Cox in the yolk sac of developing chick embryos. Although of proved protective potency in animals, the only large scale trial of this method of vaccination was during the Spanish Civil War, by Rockefeller Foundation workers. Unfortunately they were unable to remain for a period of time sufficient to evaluate their results. The use of vaccine prepared from *Rickettsia prowazeki* (the causative agent of Classical typhus) by the War Department was made standard for personnel going to the Southwest Pacific area as well as points closer to actual epidemic sites of that form of the disease. In the Australasia zone, however, the Scrub typhus or tsutsugamushi group constitutes the endemic and epidemic form. The causative organism here is the *Rickettsia orientalis*.

It is too early to state with assurance that protection has been afforded those exposed to Scrub typhus by immunization with the agent of the Classical form, and complementing this consideration is the fact that no large bodies of our troops have yet been exposed to the Classical form for estimation of the vaccine efficiency.

The inception of our study lay in the need for immunologic data with regard to typhus vaccination in human beings. The method of such study can only be by employing the Weil-Felix reaction. This procedure involves the use of *Bacillus proteus*, the Somatic (O) variety of the X19 and XK strains. The reaction of the Classical group has been to the OX19, while that of the Scrub typhus group has been to OXK. Emphasis has been laid upon the sharp distinction afforded between the groups by the use of this differential reaction. To us in this zone the problem was twofold; 1. What antibody response was being afforded by use of the vaccine. 2. To ascertain serologically the dilution at which agglutination was diagnostic of Scrub typhus.

*From the Third Medical Laboratory, Advance Section, Base Section 2, APO, Postmaster, San Francisco.
†Major, Medical Corps.
‡Captain, Medical Corps.
Received for publication, May 13, 1943.

This study was conducted upon a series of 507 sera collected at random from patients coming through our laboratory for Kahn testing. The blood was collected by venipuncture directly into sterile Wassermann tubes and clotting permitted at room temperature. The serologic testing was performed either immediately or within 24 hours. Where the interval between collection and agglutination was greater than 1 to 2 hours the specimen was placed in the refrigerator. The clot was freed by rimming with wooden applicators; and the specimen was centrifuged at 2000 revolutions per minute for three minutes. Serial dilutions in physiologic saline were made immediately. The dilutions were of the customary strength, i.e., 1:10 to 1:320, except where typhus was definitely suspected, in which case the dilutions went up to 1:2560. The dilutions noted in this paper are those of sera-in-saline, before adding antigen. The final volume of diluted serum was 0.5 c.c. in each instance.

The dilutions were made in duplicate for each serum so that reactions could be obtained against the Proteus X19 and XK ("O," or nonmotile, nonflagellated strain). To these dilutions were added 0.5 c.c. of the bacterial suspensions, prepared for us by the Department of Bacteriology. The Proteus organisms were those of a stock culture obtained from the 9th Corps Area Laboratory, Monterey, California, just prior to our departure for the Southwest Pacific Area, and carried on brain heart infusion agar. As needed, growths were subcultured on the same material and washed off with formalized saline after 48 hours. The suspension was diluted to a number three strength (barium chloride standard, 1 billion organisms). When not in use, the antigen was stored in the refrigerator.

The antigen and serial dilutions were mixed by shaking, and then were placed in the water bath (37 degrees C.) for twenty-four hours. At the end of this time readings were taken in all tubes for: (1) presence of flocculent material on the bottom of the tube, (2) the clarity of the supernatant liquid. Comparison was made in each instance with 3 controls: (1) Antigen-saline: (2) known positive serum; (3) known negative serum. Only those readings were called positive wherein there were both a heavy flocculation and complete or almost complete clarity of the supernatant before shaking. No dilution was called positive where more than an estimated 25 per cent of the antigen remained in suspension. The readings were conducted at the same hour each day, against daylight by the same reader in each instance. It can be stated that variations in all of the technical procedures from venipuncture to cleaning of the tubes was held to an absolute minimum. Only two of the enlisted men participated in the handling of this series.

FINDINGS

Our results are divisible into four categories, which we have arbitrarily set up to include all of the individuals tested. The divisions were created on the basis of relative immunologic probability. As shown in the accompanying table, these were:

1. Civilians, native to this area (except where noted).
2. Patients in the United States Army hospital units, in this area. These individuals had been immunized with the typhus vaccine at various intervals

up to a year and were in the hospital with a definite or suspected diagnosis of malaria, dengue, or syphilis.

3. Men with no clinical evidence of disease (Officer candidates), who likewise had been immunized variously less than a year before testing.

4. Patients who showed titers diagnostic of Scrub typhus.

A fifth group, those who showed positive Kahns, was incorporated into the second group, when it was seen that their responses to the Weil-Felix reaction were the same.

As can be seen in the accompanying table, the civilian group, native to this area, showed absolutely no reactivity to the Proteus either OXK or OX19. It can be stated that none of these individuals admitted having had

TABLE I

TABULATION OF RESULTS

OXK	OX19	POSITIVES	CIVILIANS	OFFICER CAND.	PATIENTS
0	0		36	22	72
1:10	0		5 (Merch. Mar.)	57	109
1:10	1:10		2 (Merch. Mar.)	14	12
1:10	1:20			4	3
1:20	0		2 (Merch. Mar.)	37	50
1:20	1:10			4	4
1:40	0		1 (Merch. Mar.)	8	17
1:40	1:10				6
1:80	0				6
1:160	0	12			
1:160	1:10	7			
1:160	1:40	5			
1:160	1:160	1			
1:320	1:0	3			
1:320	1:10	2			
1:320	1:20	3			
1:640	1:80	1			
1:1280	1:40	2			

typhus and that there were no agglutinations, even partial, among them. The eleven civilians who did show reactivity to either or both Proteus strains were all Merchant Marine personnel whose contact with the disease in World ports is highly probable. In the military personnel, immunized as previously described, only 14 per cent showed a reactivity in titers 1:40 or higher (OXK). Of these 7 per cent had reactions with the OXK in dilutions of 1:160 or higher. This last group were in each instance individuals who had a clinical diagnosis of Scrub typhus. Table I will show that although the titers against OX19 rose somewhat in these patients they were still low. The remaining number reacted variously between the 1:40 and 1:160 with the OXK. The OX19 here again were low.

The 86 per cent of the military personnel whose titers were 1:20 or less in either OX19 or OXK demonstrated some very interesting subgroupings. Foremost was the observation that 20 per cent gave no evidence of immunologic

response. The men who were in the hospital for irrelevant illnesses outnumbered the healthy group beyond their ratio within the totals. The second notation and probably the most important single factor was that 60 per cent of the immunized personnel, sick or well, gave absolutely no response to the OX19, while showing a 1:10 or 1:20 response with the OXK.

No attempt shall be made to question the use of the Weil-Felix reaction to interpret immunologic status in typhus. Since the isolation of the *Bacillus proteus* from the urine of typhus patients, agglutination against it has been the only universally accepted clinical procedure for evaluation of the response to the disease. Nor can there be any reasonable doubt as to the selective reaction by the Scrub typhus group to the XK strain, and the Classical group to the X19 strain. As stated above this study set out to utilize these procedures in a mass serologic analysis. Animal protection tests would have been the only alternative method of assay, and this would have been impossible with so many sera.

On this basis, therefore, it becomes evident that in a group of young men, certainly in good health at the time of vaccination, there is a minimal Weil-Felix response to the Cox yolk sac immunization procedure against Classical typhus. This conclusion is inescapable when it is seen that over 88 per cent of the group, whether clinically healthy or suffering from irrelevant illnesses, showed no serum agglutination at all against the OX19. The presence of low titer reactions in the remaining 12 per cent and the high titer in the one individual who admitted having had Classical typhus showed definite potency of our X19 antigen. The question arises as to whether or not the initial two-dose inoculation is sufficient to create an immune response or whether a "series of kick-up" injections are needed. On the other hand, it is most perplexing to find that 80 per cent of the military personnel, ill or well, gave some reactivity against the OXK strain, a great part of them being in the 1:20 dilutions. Since the native population showed no reaction to the Proteus, this immunity may be due to the active inoculation and not subclinical contact with Scrub typhus. Here again, the efficacy of our OXK antigen was proved by the same very high titer responses among the clinically positive group of Scrub typhus patients. The probability here is that the Rickettsia used in the vaccine was that of a strain incitive to antibody response against Proteus OXK. It is understood that workers with this vaccine have recommended a stimulating dose between six months and a year following the initial series. Yet one cannot anticipate a rise to OX19 titers even with stimulation, when the overwhelming evidence indicates lack of response at this time. The interval since inoculation in these troops was in no instance over a year, in 95 per cent less than ten months and in 55 per cent about 6 months. As we observed the accumulating results, there was no reason to attempt their statistical separation on the basis of elapsed time since immunization; all time groups were quite similar.

Our second problem is perhaps of more immediate significance and the findings therewith of less controversial nature. This refers to the titer at which agglutination shall be considered positive for the diagnosis of Scrub typhus. In keeping with the accepted interpretation of findings in the Weil-Felix reaction, OXK titers of 1:160 or higher were considered diagnostic of

the disease. In every instance these patients had been clinically diagnosed as having the disease. Where the titer was clearly 1:80, there was suspicion of the disease, but we did not feel justified in making a positive diagnosis. Titers of 1:40 were not considered as belonging to the positive group. The finding in so large a group of individuals, however, definitely show that this concept of diagnostic levels must be changed. Of our military personnel, 86 per cent gave reactions of 1:20 or less, making this titer the accepted limit of normal rather than the 1:40 and certainly not the 1:80. We present, therefore, for corroboration in the hands of other workers the serologic dilutions of 1:40 as being very suspicious of Scrub typhus, and level of 1:80 diagnostic of the disease. Whether these figures will hold for Classical typhus, we do *not* feel competent to discuss.

CONCLUSIONS

1. Using the Weil-Felix reaction as an index of immunologic status, 88 per cent of a group of military personnel immunized with the Cox classical typhus vaccine gave no response against the Proteus OX19.

2. Of this same group 80 per cent showed reactivity against Proteus OXK (the Scrub typhus strain).

3. Of the OXK reactions, 86 per cent were at a titer of 1:20, 1:10, or negative. From this we have concluded that a titer of 1:40 was very suspicious and 1:80 diagnostic of Scrub typhus.

4. The possibility is discussed of the vaccine employed being of a nature to incite immunity for Scrub typhus.

5. The problem of immunity against typhus requires additional immediate study relevant to cross reactions and specific protection afforded by vaccines.

Reference to literature other than that noted in the following bibliography was impossible due to our military situation.

We wish to thank the members of the Department of Bacteriology, under Lt. James B. O'Neill, for their assistance. The technical work was entirely done by Pfc. J. S. Carson and Pvt. R. D. Tuttle of this unit.

REFERENCES

Cilento, Sir Raphael: Tropical Diseases in Australasia, Brisbane, Q., 1942, W. R. Smith and Paterson.

Gay, Frederick P., et al.: Agents of Disease and Host Resistance, Springfield, 1935, Charles C Thomas.

Virus-Rickettsia Diseases, Cambridge, 1941, Harvard University Press.

Great Britain, War Office: Medical Diseases in Tropical and Sub-Tropical Areas, London, 1941.

Stitt, E. R., and Clough, P. W.: Practical Bacteriology, Haematology, and Animal Parasitology, Philadelphia, 1938, The Blakiston Co.

A RAPID AGGLUTINATION TEST TECHNIQUE*

Nathan Nagle, A.B., Lucille Schulze, B.S., and J. C. Willett, D.V.M.
St. Louis, Mo.

THE principle of shaking or agitation first applied practically by Kahn[1] in 1923 in his precipitation test for syphilis accounts to a large degree for the immediate results obtained by the Kahn test. Three minutes' shaking at a speed of 280 oscillations per minute with a stroke of 1½ inches (Kahn shaking machine) produces visible aggregations with beef heart lipoids (Kahn antigen) and syphilitic serum. Eagle[2] found that shaking shortened the time required for visible aggregation of antigen and specific antiserum. Visible aggregations were obtained with *Bacillus typhosus* and its antiserum in 4 minutes' and beef heart lipoids with syphilitic serum in 2 minutes' shaking. The speed or length of stroke of shaking is not stated.

The St. Louis Health Division Laboratories have used shaking in the agglutination test technique with bacterial antigens for the past several years. This practice was begun by C. L. Pfau, now deceased, but his data are not available. During the past two years sufficient data have been accumulated to show the usefulness of the method. The procedure developed in the rapid agglutination test technique for typhoid, tularemia, brucellosis, and other bacterial diseases is essentially adding 0.3 c.c. antigen suspension to 0.3 c.c. of serum dilutions, and agitating in a Kahn shaker for 6 minutes. A preliminary reading of the test is made and reported to the physician immediately if the titer is above 1:80. The test is then incubated for 5 hours at 37° C. in a water bath, after which time the final titer is reported.

Homologous antigens were added to positive sera in dilutions and agitated in a Kahn shaker for periods extending from 2 to 10 minutes. The maximum readings in the majority of sera were obtained after the 6-minute intervals. Table I shows the results with 20 sera with readings made at 2 minute intervals up to 10 and again after 5 and 18 hours' incubation at 37° C. Practically all of the sera developed their maximum titer after 5 hours' incubation.

One hundred and twenty-nine sera submitted for typhoid, tularemia, or brucellosis were tested with *B. typhosus* antigen, and readings were made after 6 minutes' shaking, 5 and 18 hours' incubation at 37° C. All sera submitted for an agglutination test are examined routinely for all three of these diseases. In a similar manner 131 sera were tested with *Bacillus tularensis,* 133 with *Brucella abortus* and 56 with *Bacillus proteus X19* antigens. Tables II, III, and V indicate the maximum titers of sera tested with *B. typhosus, B. tularensis,* and *B. proteus X19* antigens after 5 hours' incubation at 37° C. following the preliminary 6-minute shaking period. The incubation period for *Br. abortus* must be extended beyond 5 hours to obtain the maximum titer in a small percentage of sera as shown in Table IV.

*The St. Louis Health Division Laboratories.
Received for publication, June 8, 1943.

On a small number of known positive sera the rapid agglutination test method was compared with the technique described by the Standard Methods Committee of the American Public Health Association.[3] The essential differences between the two methods are shaking and length of incubation period. The A.P.H.A. method calls for 42 hours' incubation at 37° C. for *Br. abortus*,

TABLE I

EFFECT OF SHAKING AND INCUBATION OF ANTIGEN-SERUM MIXTURES ON TITERS OF 20 SERA

SERUM WITH HOMOLOGOUS ANTIGEN	NO.	SHAKING TIME IN MINUTES					INCUBATION AT 37°C. IN HOURS	
		2	4	6	8	10	5	18
B. typhosus	1	–	20	40	40	40	40	40
B. typhosus	2	–	20	40	40	80	80	80
B. typhosus	3	20	20	20	20	20	20	40
B. typhosus	4	20	20	40	40	40	40	40
B. typhosus	5	20	40	40	80	160	160	160
B. typhosus	6	20	40	80	80	160	160	160
B. typhosus	7	20	20	40	40	80	80	160
B. typhosus	8	40	40	80	80	80	80	80
B. typhosus	9	320	320	640	640	1280	1280	1280
B. typhosus	10	1280	1280	1280	1280	1280	1280	1280
B. tularensis	11	40	80	80	80	80	80	80
B. tularensis	12	320	320	1280	1280	1280	1280	1280
B. tularensis	13	640	1280	1280	1280	1280	1280	1280
B. tularensis	14	640	1280	1280	1280	1280	1280	1280
B. tularensis	15	640	1280	1280	1280	1280	1280	1280
Br. abortus	16	160	320	640	640	640	1280	2560
Br. abortus	17	320	1280	1280	1280	1280	2560	2560
Br. abortus	18	640	1280	1280	1280	1280	1280	1280
Br. abortus	19	1280	1280	2560	2560	2560	2560	2560
Br. abortus	20	1280	1280	5120	5120	5120	5120	5120

TABLE II

EFFECT OF SHAKING AND INCUBATION OF *B. typhosus* ANTIGEN-SERUM MIXTURES ON TITERS OF 129 SERA

NO. SPEC.	6 MINUTE SHAKING	5 HRS. INCUBATION	18 HRS. INCUBATION
108	–	–	–
3	–	20	20
7	20	40	40
2	20	80	80
2	40	40	40
2	40	80	80
2	320	640	640
2	640	640	640
1	640	5120	5120

TABLE III

EFFECT OF SHAKING AND INCUBATION OF *B. tularensis* ANTIGEN-SERUM MIXTURES ON TITERS OF 131 SERA

NO. SPEC.	6 MINUTE SHAKING	5 HRS. INCUBATION	18 HRS. INCUBATION
112	–	–	–
3	20	20	20
2	20	80	80
1	80	80	80
4	160	160	160
1	160	320	320
1	320	640	640
2	640	640	640
3	1280	1280	1280
1	2560	2560	2560
1	5120	5120	5120

18 hours at 52° C. for *B. typhosus,* and 2 hours at 37° C., and overnight at 8° C. for *B. tularensis* and *B. proteus.* Table VI shows the close agreement between the results obtained on 36 sera.

TABLE IV

EFFECT OF SHAKING AND INCUBATION OF *Br. abortus* ANTIGEN-SERUM MIXTURES ON TITERS OF 133 SERA

NO. SPEC.	6 MINUTE SHAKING	5 HRS. INCUBATION	18 HRS. INCUBATION
115	-	-	-
1	-	40	80
1	20	320	320
3	80	160	160
1	80	80	160
1	160	320	320
1	640	1280	2560
1	640	2560	2560
1	1280	2560	2560
1	1280	1280	1280
1	1280	1280	2560
2	1280	5120	5120
1	2560	5120	5120
2	5120	5120	5120
1	40,960	81,920	81,920

TABLE V

EFFECT OF SHAKING AND INCUBATION OF *B. proteus X19* ANTIGEN SERUM MIXTURES ON TITERS OF 56 SERA

NO. SPEC.	6 MINUTE SHAKING	5 HRS. INCUBATION	18 HRS. INCUBATION
31	-	-	-
2	-	20	20
1	-	40	80
4	20	20	20
4	20	40	40
4	40	40	40
2	40	80	80
4	80	80	80
2	320	320	320
2	640	1280	1280

The agglutination tests recorded in Table VI for *B. typhosus* and *Br. abortus* done by the A.P.H.A. method were also read after 6 minutes' incubation at room temperature, and again after 1, 2, and 5 hours at either 52° C. or 37° C. Visible aggregations were not observed after the 6-minute period although a small number showed aggregations in low dilutions after 1-hour incubation. The low titers obtained after 5 hours' incubation showed that long incubation periods are necessary in order to obtain the maximum titer when the antigen-serum mixtures are not shaken. Duplicate tests done by the rapid method were also read after 6 minutes' shaking and again after 1, 2, and 3 hours' incubation in the water bath at the appropriate temperature. Immediate reactions in low dilutions following the 6-minute shaking period were obtained with practically all sera recorded in Table VI. Higher titers were obtained after 3 hours' incubation, although it was necessary to incubate an additional 2 hours to obtain the maximum titer.

Antigen preparation in this laboratory follows the A.P.H.A. method very closely except that for Brucella antigen, formalin is used instead of phenol and the turbidity is 500 parts per million instead of 200 parts per million. The cul-

tures used are those distributed by the National Institute of Health including *B. typhosus* "O" and "H," *B. tularensis, Br. abortus,* and *B. proteus.* Sera submitted for Typhus or Rocky Mountain Spotted Fever are first examined with a *B. proteus X19* culture composed of both motile and nonmotile organisms made into a 0.3 per cent formolized suspension with a turbidity of 500 parts per million. Sera showing at least a 1:40 titer are repeated with live culture antigens made from *B. proteus OX19* and *OX2.* Table VI shows that the formolized and live culture antigens are equally sensitive.

TABLE VI

THE RAPID AGGLUTINATION TEST COMPARED WITH THE AMERICAN PUBLIC HEALTH ASSOCIATION METHODS ON 36 SERA

NO. SERA	RAPID METHOD 6 MINUTES' SHAKING 5 HOURS' INCUBATION	A.P.H.A. METHODS
Typhoid		
2	20	20
3	80	40
1	160	40
1	160	160
1	320	320
1	320	80
1	640	320
1	1,280	1,280
1	10,240	10,240
Tularemia		
1	80	80
1	1,280	640
1	1,280	320
1	1,280	1,280
1	2,560	2,560
Brucellosis		
2	1,280	1,280
2	2,560	1,280
1	5,120	2,560
1	10,240	10,240
Typhus		
1	20	20
3	20	40
1	40	40
2	80	80
2	80	40
1	80	160
1	160	160
1	1,280	1,280
1	2,560	5,120

CONCLUSIONS

The inclusion of shaking in the usual agglutination test technique enables the laboratory to secure comparatively rapid results. Six minutes' agitation in a Kahn shaking machine of the antigen-serum mixtures permits preliminary positive results to be reported at once, and following 5 hours' incubation at 37° C., the final titer can be reported.

REFERENCES

1. Kahn, R. L.: Serum Diagnosis of Syphilis by Precipitation, Baltimore, 1925, Williams and Wilkins Co.
2. Eagle, H.: Specific Agglutination and Precipitation. II Velocity of the Reactions, J. Immunol. 23: 153, 1932.
3. Diagnostic Procedures and Reagents, New York, 1941, Am. Pub. Health Assoc.

CRITERIA FOR THE SELECTION OF AN ELECTROENCEPHALOGRAPH*

Louis W. Max, Ph.D.,† Leo Wiesner, Ph.D.,‡ and
Jesse G. M. Bullowa, M.D.,§
New York, N. Y.

The history of electrophysiology has repeatedly shown that experimental results may be distorted by the physical limitations of the recording instruments. This danger is especially marked in the comparatively new field of electroencephalography, where standardization of apparatus and procedure has not kept pace with the pressure for clinical applications. Accordingly, the relative advantages and disadvantages of available electroencephalographs merit careful scrutiny, since an error in judgment at the time of procurement may result in erroneous experimental and clinical conclusions.

Physical factors determining the accuracy of instruments for physiologic work and illustrations of errors introduced by inferior recorders have already been dealt with at some length by one of us:[1] the present report applies the earlier discussion to the selection of an adequate brain wave recorder.

ESSENTIAL REQUIREMENTS

1. *Constant Frequency Response.*—Among the requirements an electroencephalograph must fulfill in order to record an accurate picture of the brain potentials is constant frequency response. This means that individual brain waves of equal voltage even though of different frequencies must appear in the record as equal in height or amplitude. If the instrument cannot record the voltage of different frequencies with equal fidelity, certain waves of clinically significant frequencies may be discriminated against and escape detection.

2. *Linearity of Voltage Response.*—Another important requirement is linearity of amplitude response. The height of the pen deflections must be directly proportional to the magnitude of the brain potentials. For satisfactory recording and proportionality, this should hold within 5 per cent for pen displacements up to 7.5 millimeters on either side of the zero line.

Testing Apparatus: The following apparatus arrangement (Fig. 1) was employed to check upon the accuracy of frequency and amplitude re-

*From the Medical Service, Dr. Oswald LaRotonda, Director, of Harlem Hospital, Department of Hospitals.

†Associate Professor of Psychology, New York University, Littauer Fellow in Pneumonia Research, Harlem Hospital, New York City, Visiting Professor of Physiology, New York University College of Dentistry.

‡Physicist, Littauer Pneumonia Research Fund, Harlem Hospital, New York City.

§Clinical Professor of Medicine, New York University College of Medicine; Visiting Physician, Harlem Hospital, New York City; Visiting Physician, Willard Parker Hospital.

From the Littauer Pneumonia Research Fund of New York University, College of Medicine. These studies received additional support from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

Received for publication, June 9, 1943.

sponses of three commercial varieties of electroencephalograph. An electronic oscillator generates electrical waves of variable frequency; these waves are impressed upon a calibrated attenuator which adjusts their voltage to an appropriate value; these measured waves are, in turn, supplied simultaneously to the several channels of the electroencephalograph under test (EEG) and to an Einthoven electrocardiograph (ECG). The latter provides an independent, photographic check on the wave form, frequency, and amplitude of the test waves. The Einthoven galvanometer was adjusted to a constant frequency response from 0 to 100 cycles per second (c.p.s.).

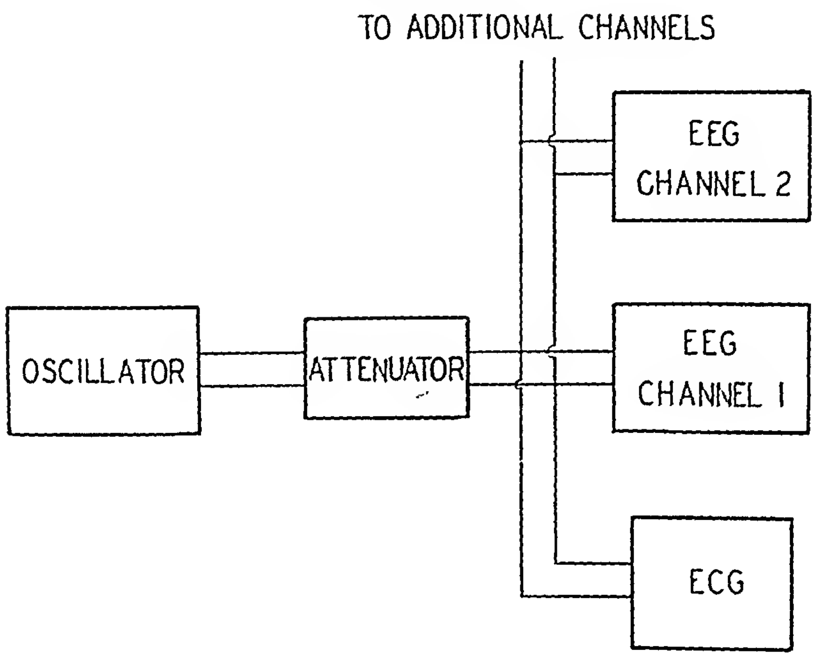


Fig. 1.

Fig. 2 presents the results of the above test on the three varieties of electroencephalographs tested. The curves present, in millimeters, the responses of the recording pens to electrical waves constant in amplitude but varying in frequency from 1 to 100 c.p.s.

It will be noted that while none of the recorders has a flat response in the entire range, the curves from two of the recorders, designated A and B, are markedly irregular in the range from 1 to 10 cycles, a range of frequencies of marked clinical importance. All of the curves are relatively flat in the range from 10 to 20 cycles, but instrument A showed an undesirable resonance peak at 25 cycles. Since this peak is well within the range of the physiologic frequencies to be recorded, serious frequency distortion would result.

In amplitude response, none of the models proved precisely linear, but the irregularities were not as important as in the case of the frequency response. Even the best of the three recorders deviated from strict linearity by a maximum of 13 per cent ($\pm$ 6.5 per cent).

3. *Freedom From Temperature and Humidity Effects.*—It is important that variations of temperature and humidity encountered in the average

laboratory shall not interfere with the stability of the recorder's operating characteristics. One of the instruments tested, model B, showed varying frequency and amplitude responses on different days; these, we discovered, were a function of its proximity to a warm radiator. This model was tested on a rolling laboratory table, and its stability of response depended on its distance from the radiator. Model A was free of this temperature effect, but its writing pens failed to maintain a constant zero position on moist days. Model C remained unaffected by such temperature and humidity variations.

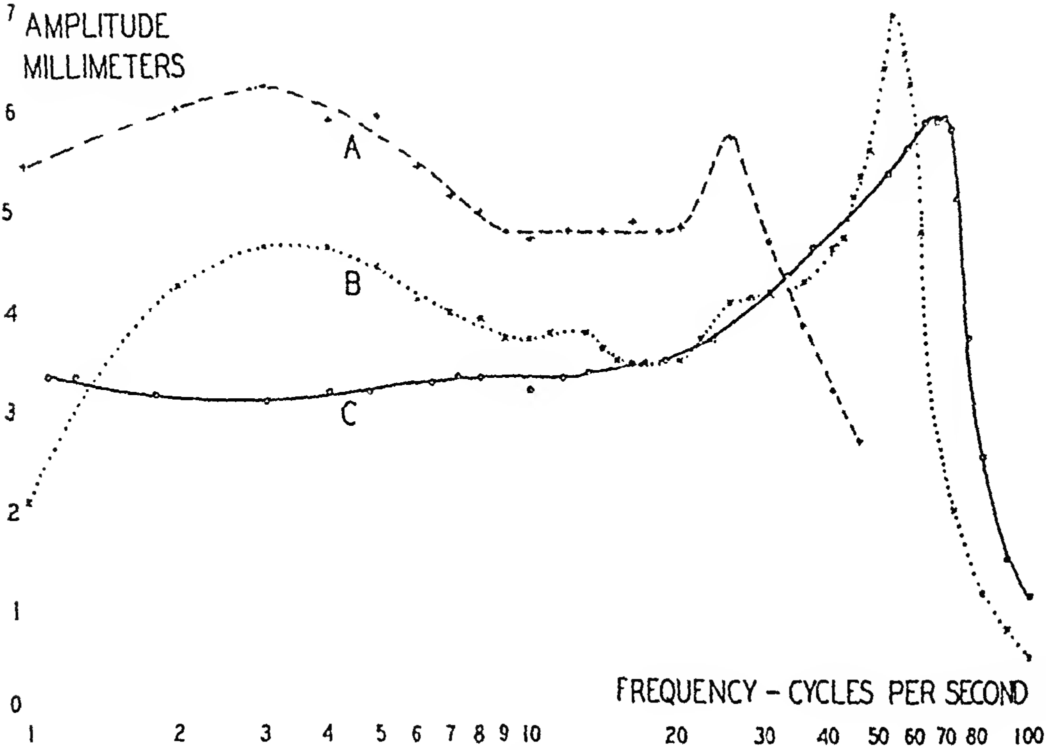


Fig. 2.

The following requirements seemed to be adequately met by all three of the models studied:

4. *Identity of Component Channels.*—Where, as is usually the case, a multiple channel electroencephalograph is to be purchased, the channels must be adjustable so that identical input voltages will produce identical records on all channels. This is important because it is sometimes desirable to switch electrode leads from one channel to another in the course of an electroencephalographic examination. Hence, a multiple channel instrument should meet the following test: After all the channels have been adjusted to give equal pen deflections for equal input voltages, all the channel inputs are to be connected in parallel to the same two electrodes on the patient's skull and simultaneous tracings taken. The tracings may then be superimposed on each other and no two of them should differ by more than ±5 per cent. If the amplifiers or recording pens are not well matched, the instrument will not pass this test.

5. *Freedom From "Cross Talk."*—One channel must not interfere with or influence the others. Such interference, termed "cross talk" by analogy with a similar disturbance in telephone and radio communication, makes the records worthless.

One way to check this is to connect one channel to the patient's skull; connect the inputs of the remaining channels to a noninductive 5000 ohm resistor and adjust them for maximum amplification. No component of the brain wave record of the first channel should appear in the other channels.

6. *Low Noise Level.*—Where no voltage is applied to the input of the electroencephalograph, one would expect a zero deflection or straight line to be recorded. Actually the line will probably contain some slight fluctuations termed "noise." Since brain waves of an amplitude lower than these random fluctuations cannot be detected, the "noise level" should be as low as possible and should not in any event exceed 2 microvolts. To check this, connect the inputs of all the channels to a noninductive 5000 ohm resistor (to simulate the patient's resistance); adjust the amplifiers for maximum gain and inscribe a record. As estimated from the microvolt calibration, the noise fluctuation should remain below the two microvolt level. This is particularly important for instruments containing B batteries, since the adventitious noise will increase as the batteries age and will later prevent satisfactory recording.

In view of the optimistic claims of manufacturers, purchase orders for an electroencephalograph should include definite specifications in accordance with the above requirements.

SUMMARY

Criteria for the adequacy of an electroencephalograph are described, and tests are suggested for checking its performance. Frequency response curves of three commercial instruments are presented.

REFERENCE

1. Max, L. W.: An Experimental Study of the Motor Theory of Consciousness I. Critique of earlier studies, J. Gen. Psychol. 11: 112-125 (cf. especially pp. 117-122), 1934.

SOME EXPERIMENTS ON THE ROMANOVSKY STAINING OF BLOOD FILMS

R. D. LILLIE, M.D.,* BETHESDA, MD.

IN the course of testing samples of Wright and Giemsa stains, it became necessary to arrive at a technique which would give the best tinctorial results of which the sample was capable, regardless of time or of convenience of the method, and these experiments led to results which appear to be of general practical interest.

Controlled experiments were used, using several samples of dye, varied staining times, and varied delutions. As test objects thin blood films from white rats heavily infected with *Trypanosoma equiperdum* were used. These were fixed as soon as dry in absolute methanol for 2 minutes. A phosphate buffer of pH 6.5 was used throughout, as this gives a good orange-pink color to erythrocytes and satisfactory blue cytoplasm to lymphocytes. The stock buffer contained 6.2 Gm. KH_2PO_4 and 3.0 Gm. Na_2HPO_4 in 500 c.c. each of CP methanol and distilled water and was diluted 1:20 with distilled water for use.

In the first experiment 1/2 and 1/3 dilutions of Wright stains LWr-17 and NWr-18 were used for 1, 2, 4, 7.5, and 15 minutes; 1/5 dilution for 2, 4, 7.5, 15, and 30 minutes; 1/10 for 4, 7.5, 15, 30, and 60 minutes; 1/20 and 1/32 for 7.5, 15, 30, 60, and 120 minutes, and 1/50 for 15 and 30 minutes, 1, 2, and 4 hours. The dilutions were made in test tubes and thoroughly mixed, as this procedure was found to give more uniform staining throughout the smear than the traditional method of mixing on the slide.

The 1/2 dilution, that is, one part of methanol stain solution and one of buffered water, gave fair staining of nuclei in blue to violet tints in 4 to 15 minutes, but the desired reddish purple chromatin staining was not attained. This coloration appeared first with the 1/3 dilution in 4 to 15 minutes, best at 7.5, rather dark in 15, but was best with the 1/5 and 1/10 dilutions in 7.5 to 30 and 15 to 60 minutes respectively. Satisfactory staining was still attained with the 1/20 dilution in 1 and 2 hours, but with higher dilutions did not occur even in 4 hours. The optimal dilutions and times of those tried were 1/5 for 15 minutes and 1/10 for 30 minutes.

Next, a similar series of stains was made with two samples of Giemsa stain, NGe-13 and NIH-2B, using 6 adjacent time intervals out of the following series: 2, 4, 7.5, 15, 30 minutes, 1, 2, 4, and 6 hours, for each dilution. The dilutions used were 1/10, 1/17, 1/25, 1/36, 1/50, 1/75, and 1/100. The two samples gave fairly similar results, with a slight but consistent difference in favor of the second. Optimal staining gave deep red purple leucocyte and lymphocyte nuclei, clear medium blue or slightly greenish blue cytoplasm of lymphocytes and trypanosomes, and sharply stained trophonuclei, blepharoplasts, and un-

*Senior Surgeon, U. S. Public Health Service, Division of Pathology, National Institute of Health.

Received for publication, June 17, 1943.

dulating membranes. It was noted in this and all subsequent experiments that when trypanosome staining was optimal, lymphocytes were somewhat overstained, while with optimal lymphocyte staining undulating membranes were often indistinct.

Times required for optimal staining with the various dilutions were as follows: 1/10, 15 to 60 minutes; 1/17, 1/25, and 1/36, 30 to 60 minutes; 1/50 and 1/75, 1 to 2 hours; and 1/100, 2 to 4 hours.

Following our experience with the acceleration of azure eosinate staining of tissues with acetone,[1] it was determined to apply this method to Giemsa staining of blood films. Accordingly, eleven 40 c.c. quantities of 1:50 dilution buffered to pH 6.5 were made up, using Giemsa stain NIH-2B and containing 0 to 10 c.c. acetone by 1 c.c. steps. Five staining intervals were used with each mixture, spaced so that each successive interval was approximately twice as long as the previous one.

Good but not quite optimal staining was attained in one hour without acetone. Optimal staining was attained in one hour with 1 c.c. acetone, in 30 minutes with 2 or 3 c.c. acetone, in 15 minutes with 3 or 4 c.c. acetone; good but suboptimal staining in 30 minutes with 5 or 6 c.c. acetone; inferior staining in any interval with 7 or 8 c.c., and very poor with 9 or 10 c.c.

As 3 c.c. acetone to 40 c.c. dilute stain seemed to be about the optimal quantity, it was decided to determine the effect of various dilutions of stock Giemsa NIH-2B for various time intervals, with and without acetone.

Five dilutions were used: 0.8 c.c., 1.0, 1.2, 1.4, and 1.6 c.c. stock stain to a total of 40 c.c. diluted mixture, (ranging from 1/50 to 1/25 dilution), with and without 3 c.c. acetone in the mixture. Four time intervals were used, each double the preceding as before. Optimal staining times for the 5 dilutions without acetone were 1 to 2 hours for the first two dilutions, 30 to 60 minutes for the remaining three. With acetone, times were 30 to 60 minutes for the first 2 dilutions; 15 to 30 minutes for the next two; and 15 or less for the last. Similar results were attained on repetition of this test using Giemsa stains NIH-2B, NGe-13, and LGe-6 at 1/50 dilution without acetone and 1/25 with acetone. The acceleration of staining in favor of the latter was between 3 and 4 times.

As these tests indicated that more concentrated dilutions of Giemsa stain might give even faster staining, a further series of tests was made using 2, 2.5, 3, 3.5, and 4 c.c. Giemsa stain LGe-6, 3 c.c. acetone and water buffered to pH 6.5 to make 40 c.c. Time intervals used were 2.5, 5, 7.5, 10, 15, 20, and 30 minutes. Optimal staining of trypanosomes and blood cells was attained in 2.5 to 5 minutes with the 1/10 dilution, while about 10 to 15 minutes was needed with the weakest (1/20) dilution.

Repeating this experience on a limited number of unfixed thick and methanol-fixed thin human blood films containing *Plasmodium vivax*, stains comparable to those obtained in 30 to 60 minutes at 1:50 dilution without acetone were attained in 15 to 20 minutes at 1:25 with acetone, and in 5 minutes at 1/10 dilution. The Giemsa (N43) used in this test contained some relative surplus of methylene blue, so repetition of the test with three other samples, NIH-2A, LGe-6, and NGe-13, was done, using thick and thin films containing *Plasmodium malariae*. Again, acceptable staining of parasite chromatin was attained in 6 minutes in both thick and thin films with all three samples and with one in 4

minutes. The thick films were prepared in the morning and stained the same afternoon. It is noteworthy that of 21 thick films stained 2 to 16 minutes, none were lost, while of 4 control thick films stained 30 to 90 minutes in 1:50 Giemsa without acetone, only one had a small area left to look at on the slide.

In the course of these experiments it was noted that one series fixed with previously used methanol seemed to give slightly better staining than expected. Thinking that this might be due to the presence of some water in the used methanol, experiments were made using 100 per cent, 95 per cent, 90 per cent, 85 per cent, 80 per cent, 70 per cent, 60 per cent and 50 per cent methanol in distilled water. Two lots of Giemsa were used, NIH-2B and LGe-6, 1/50 dilution without acetone, and 1/25 with 3 c.c. acetone to 40 c.c. with the first, the latter only with the second.

The 50 per cent and 60 per cent methanol gave complete hemolysis. Much karyolysis also was evident with 50 per cent methanol, fairly good leucocyte and rather poor trypanosome staining with 60 per cent. With 70 per cent some red corpuscles were intact, others partly hemolyzed, and with 80 to 100 per cent methanol red corpuscles were well fixed. There was no appreciable difference in the minimum time required for optimal staining of lymphocytes, leucocytes, and trypanosomes with methanol percentages from 70 to 100 per cent. Prolonging the fixation interval in 90 to 100 per cent methanol from 2 to 10 minutes appeared to improve the staining somewhat.

The Giemsa stain NIH-2B, which was the one most used in these experiments and appeared to be equal to or better than the commercial samples, was made in our laboratory from methylene blue and eosin by the method recently reported.[2] The procedure is quoted as follows, since the original may not be available to hospital laboratories.

"Dissolve 10 gm. methylene blue of 85-88 percent dye content in 600 cc. distilled water. Add 6.8 cc. concentrated sulfuric acid (sp. gr. 1.835 to 1.84). Bring to a boil and add 2.5 gm. potassium bichromate dissolved in 25 cc. distilled water. Boil 20 minutes. Cool to 10° C. or lower (place in refrigerator over night). When cold add 17.5 gm. sodium bicarbonate slowly with frequent shaking. Then add a 5 percent solution of eosin Y of about 90 percent dye content and shake constantly until margin of fluid appears pale blue or bluish pink. About 205 cc. will be required and three-fourths of this can be added at once. Filter at once, preferably on vacuum funnel with hard paper. When fluid has been drawn through and surface begins to crack, add 100 cc. distilled water, let drain, and wash again with a second 100 cc. distilled water. Lay the (opened out) filter on a larger piece of filter paper or paper towel and dry overnight on warm plate or in incubator, at 37°C. The drying may be accelerated by using two 100 cc. portions of acetone or, preferably, 95 percent alcohol as washes after the second wash with water. Drying at 55° to 60° C. has been tried, and produces quite a little alteration of the thiazin dye; less if acetone or alcohol washes are used and the heating limited to 2 or 3 hours. This is the crude azure B eosinate.

"To make the crude azure A eosinate, proceed exactly as above but take 5 gm. potassium bichromate in place of 2.5 gm. and dissolve it in 50 cc. distilled water.

"To make the methylene blue eosinate, dissolve 10 gm. methylene blue in

600 cc. cold distilled water and precipitate as before with 5 percent eosin, filtering and drying as above."

"To make the finished stain, grind the three eosinates separately into fine powder in separate clean mortars. (The same mortar may be used if washed out with water, dried with a paper towel, washed with concentrated sulfuric acid until no more green color is liberated, then again with water and alcohol.) Then weigh out 500 mg. crude azure B eosinate, 100 mg. crude azure A eosinate, 400 mg. methylene blue eosinate, and 200 mg. finely ground methylene blue. Decant the mixed powder onto the surface of 200 cc. of solvent allowing it to settle in gradually. Then shake frequently for 2 or 3 days, keeping the bottle between 50° and 60° C. between shakings.

"The traditional solvent is equal parts of glycerin and methyl alcohol. If the bottle is tightly stoppered and the fluid level marked on the outside with a grease pencil or a piece of adhesive, there will be little or no loss from evaporation, and in any case the fluid level can be restored by addition of methyl alcohol. The glycerin should be neutral, anhydrous, and of the purest grade obtainable. If the special methyl alcohol for blood work is unobtainable, ordinary C.P. methonal may be repurified by distillation in glass after adding 4-5 gm. each of silver nitrate and sodium hydroxide. This destroys aldehydes and anchors volatile acids as sodium salts."

CONCLUSIONS

Wright stain solutions made up in methanol in the usual 125 mg. per 100 c.c. proportions can be made to give blood and parasite stains comparable in color effects to Giemsa by increasing the dilution to 1/5 to 1/10 and prolonging the staining interval to 15 to 30 minutes.

Previous fixation in 80 to 100 per cent methanol and staining with premixed dilutions of either Wright or Giemsa stain is the preferable procedure. This indicates that methanol may be used repeatedly for fixation, as long as no evident hemolysis is produced.

Addition of 5 to 10 per cent acetone, best 7.5 or 3 c.c. to 40, decreases the time required for Giemsa staining to about half. Increase in the concentration of Giemsa stain in the final staining mixture decreases the required time proportionately so that with a 1:10 Giemsa stain dilution containing 7.5 per cent acetone excellent stains of thin blood films are obtained in 5 minutes, and thick films may be adequately stained in 5 to 6 minutes in the same mixture. Further, thick films may be stained promptly with much less danger of losing them when such short intervals are employed.

REFERENCES

1. Lillie, R. D.: On the Use of Acetone and Methanol in Romanovsky Staining of Tissues. In press, Stain Technol.
2. Lillie, R. D.: A Giemsa Stain of Quite Constant Composition and Performance, Made in the Laboratory From Eosin and Methylene Blue, Pub. Health Repts. 58: 449, 1943.

THE RECORDING OF LUNG CONTRACTIONS BY A PULLEY AND LEVER METHOD*

D. E. Jackson, M.D., Cincinnati, Ohio

A VARIETY of methods have been used by different investigators to study the reactions of the bronchioles to drugs, to electrical stimulation of nerves, blood pressure changes, air pressure variations, and other phenomena connected with the functions of the lungs and the respiratory air passages. The lungs and the air passages have such varied and extensive functions to perform that no single method could be expected to cover all phases of these phenomena. Earlier workers have placed a lobe of one lung in a plethysmograph (Brodie and Dixon, M. Cloetta) or have used a metal plate ("lung shield") in the chest to form a plethysmograph for one lung (Jackson), or a "lung plethysmograph" was placed inside the chest[1] to hold the walls in a relatively fixed position while the chest cavity was intermittently aspirated to carry on artificial respiration, the animal usually being pithed (Jackson). Another method involved the insertion of a cannula into the chest cavity, or the passage of a perforated tube transversely through the anterior portion of the chest wall, thus permitting air pressure changes from both pleural cavities to be recorded. More recently many workers have studied the reactions of small rings of isolated sections cut from the bronchioles. Sollmann and his co-workers have perfused isolated lungs through the trachea and noted the rate of outflow through perforations in the lungs. They have also used a special method to make microscopic cinematic studies of cross sections of fresh bronchioles.[2]

Most of these methods are too complicated or too time-consuming to be easily used by ordinary beginning medical students. Recently I have been using a method which I believe may be readily employed in ordinary class work.

Fig. 1 illustrates the apparatus and the technique which I have used. The animal (dog) is anesthetized and placed on the operating table. The chest is opened by a mid-line incision, the sternum being sawed through endwise, and four strong ligatures about eighteen inches long are passed through the edges of the opening in the chest and tied around the sawed edges of the sternum. These ligatures usually shut off the internal mammary arteries and veins and thus check any bleeding from cut branches of these vessels. At the upper end of the opening in the chest the mammary arteries should be examined and separated down to their origins, for in some animals these arteries branch off from a single stem. If this condition is found, then one of the arteries should be doubly ligated close to its origin and the artery should then be cut between the ligatures. The chest should now be pulled open about three or four inches and held in this position by tying the ligatures at the side of the dog board. If

*From the Department of Pharmacology of the University of Cincinnati College of Medicine.

Received for publication, September 9, 1943.

the chest is thus pulled open in an animal in which the mammary arteries come off from one stem, one of them may be torn off unless it has been tied and sectioned previously.

Positive artificial respiration is used, the lungs being well but not excessively inflated at the rate of about twenty-five or thirty times per minute (dog). The lungs and heart are freed from the membranous anterior mediastinum, and care is taken to see that all lobes move freely in the chest and over the heart. A string about four feet long which is as free as possible from stretch (a fishing line is very satisfactory) is now attached by a stitch to the tip of one lobe of the lungs. I have generally used the upper right lobe, but in some cases other lobes may be used to advantage.

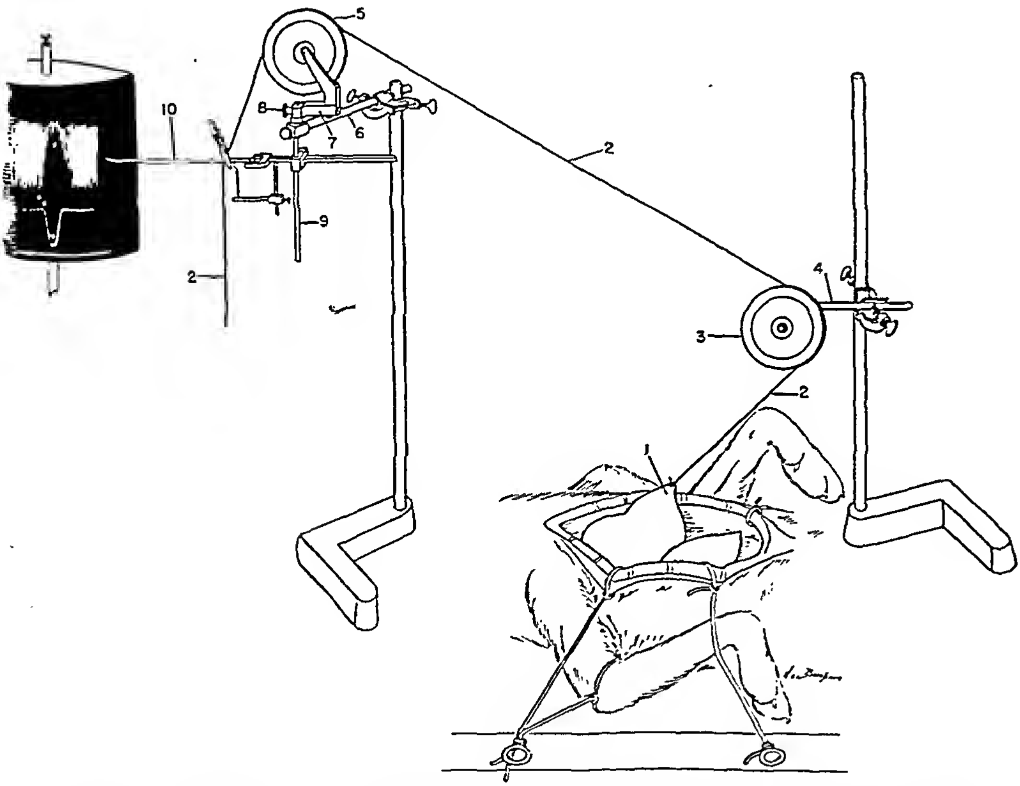


Fig. 1.—Diagrammatic representation of the lung apparatus and its method of application. For discussion, see text. (Apparatus made by the Max Wocher & Son Co., Cincinnati, Ohio.)

In Fig. 1 the string (*2*) is attached to the tip of the right upper lobe of the lung. The string passes over a pulley (*3*), four inches in diameter, and thence to the second pulley (*5*), also four inches in diameter. The distal end of the string is then clamped to the writing lever by a small, light bulldog clamp, which allows the string to be quickly and easily adjusted to give the desired magnification by the lever, or to make the lever write higher or lower on the drum. The two pulleys are made of light wood but have metal hubs which turn very freely on metal spindles. The pulleys are made large so that they can be turned very easily and thus not hamper the movements of the

lung lobe. The pulley *3* is supported by the rod *4*, which can be turned in the clamp on the stand. But the end of the rod *4* is bent at a right angle (behind the pulley *3*) and this short bent end turns in the metal block which carries the spindle supporting the pulley. This adjustment is secured by a setscrew in the metal block. By these two adjustments the pulley can be placed in any position and made to rotate in a plane which will direct the string toward the second pulley *5*, and also in such a position that the string attached to the lung can pull in the proper direction to record the movements most advantageously. The pulley *5* has some six different adjustments which are attained by the special arrangements shown in the parts *6*, *7*, *8*, and *9*. The writing lever (*10*) also has three adjustments, up and down (on the rod *9*), circularly (on the rod *9*), and back and forth (through the block and setscrew attached to rod *9*). The tension of the writing lever is partly regulated by a very fine, adjustable, coil spring and partly by the weight of the small bulldog clamp.

The writing point itself is made of thin aluminum foil so that it is light and very flexible. Since it moves at a rather rapid speed (25 to 30 double strokes per minute), it should be just stiff enough to maintain good contact with the surface of the drum. The up and down stroke on the drum should be about three and one-half to four inches (dog). It is essential that the whole mechanism works smoothly and with a minimum of friction. It is generally necessary, especially in the beginning, to do a little experimenting to find just the amount of weight and magnification to use for the lever. It can be seen that the device has a good number of adjustments. I have found that the proper adjustment is very necessary in order to get the optimum effects from the lung, and this is one of the most likely points for failure, even though the technique for the rest of the experiment has been entirely correct. At such a time I have repeatedly found that some slight readjustment of the apparatus may lead to complete success.

To obtain the best results, there will generally be two fundamental requirements which must be met. These are, first, a perfectly reliable artificial respiration machine, and, second, the animal must be kept still. Most laboratories now are equipped with respiration machines which are motor-driven and will regularly deliver constant quantities of air under constant pressure for each inspiration. An ordinary T-form of tracheal cannula is used, and the excess air delivered by the machine at each discharge is allowed to escape from the cannula through a short piece of rubber tubing which carries an adjusting (Hoffman's) screw clamp. In this manner the desired extent of lung inflation is easily secured.

Fig. 2 shows the effect which stimulation of the vagi nerves has on the lungs. Movements were recorded from the right upper lobe, but stimulation of either the right or the left vagus nerve in the cervical region caused a contraction. But the right vagus seems to be a little more effective than the left on the right lung lobe. Apparently the two vagi have so completely anastomosed before they pass into the lungs that either nerve can control either lung. This follows the embryologic development of the vagi along the gastrointestinal tract and the branching off of the lungs from the primitive gut. No doubt this is important clinically. There are some slight variations in the response of the lung to vagus stimulation, but these apparently follow very

closely the similar variations in cardiac response to the same stimulation. And the vagi endings in the lungs can be readily fatigued by stimulation just as occurs in the heart.

Fig. 2 also illustrates the marked increase in general tone of contraction of the lung lobe as shown by the whole tracing tending to rise above the normal level as the nerves are stimulated. This is also shown in another way in Fig. 4.

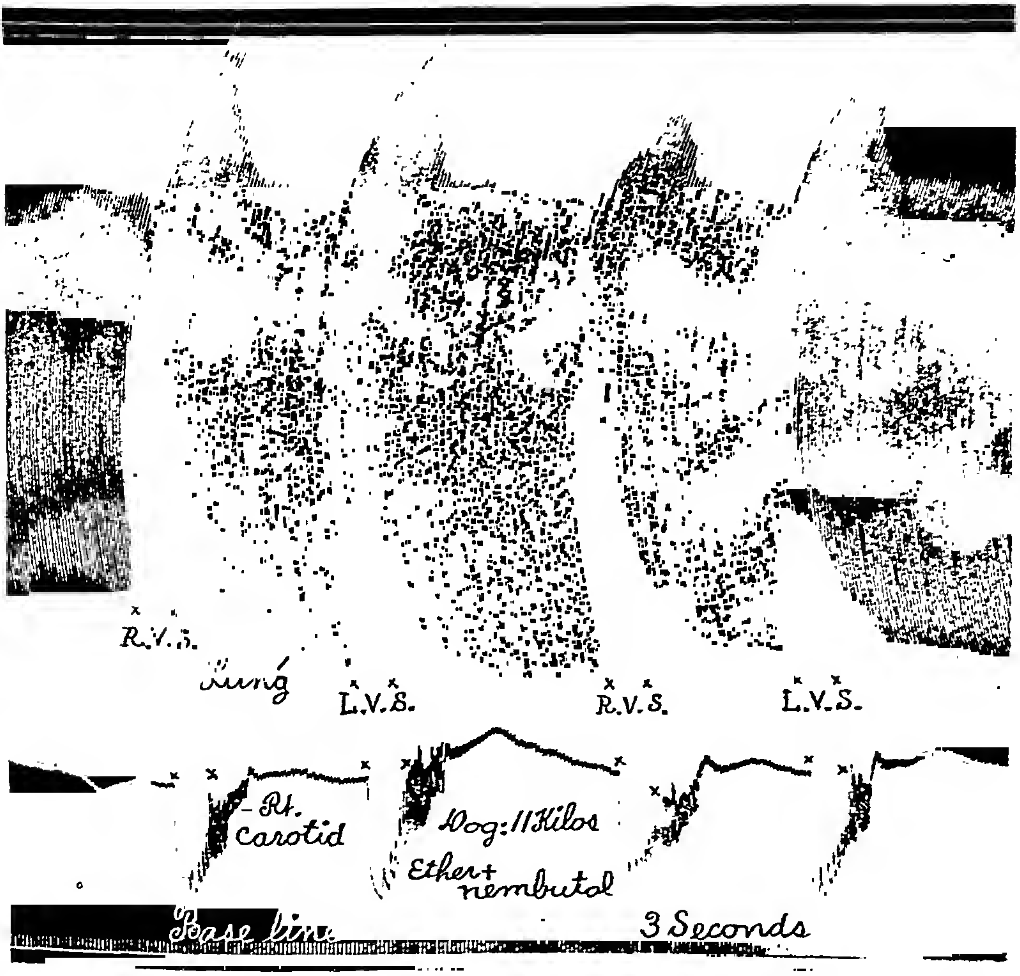


Fig. 2.—Lung tracing made from the upper lobe of the right lung, and the blood pressure recorded from the carotid artery. R. V. S., right vagus stimulated. L. V. S., left vagus stimulated.

The second basic requirement, that of keeping the animal still, may be attained in different ways. A drug of the curare type, or a large dose of nicotine, might be used. But in many cases these would be objectionable because of the ganglionic action which effective doses of these drugs may have. This might obscure or prevent entirely any central action which a drug injected later might have on the lungs. I have generally succeeded very well in keeping the animal quiet by giving ether only until all operative procedures

were completed, and then I have injected intravenously one or more cubic centimeters of nembutal solution (Abbott's solution). This is done slowly and cautiously until just the right depth of anesthesia is obtained, and then a normal tracing is taken and the drug to be studied is injected, or such other procedures as have been planned are carried out. In a certain number of instances some disturbances of the records may occur, because a profound bronchoconstriction or a great fall in blood pressure may lead to such a marked degree of asphyxia that this leads to central stimulation and may

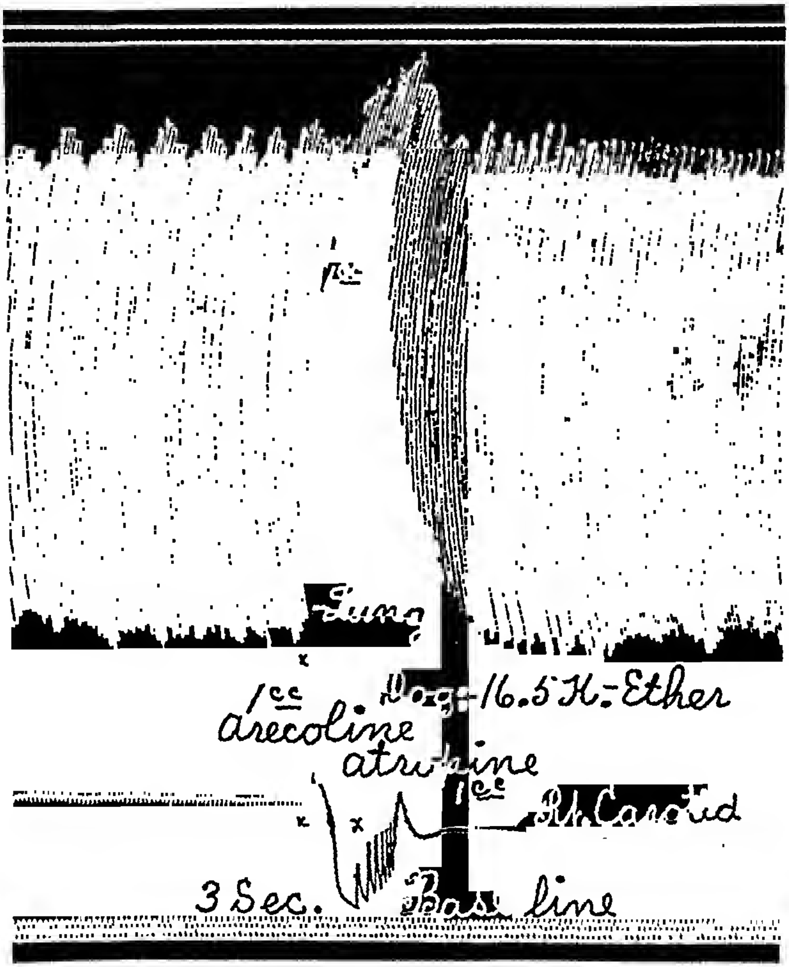


Fig. 3.—Lung tracing and blood pressure showing the action of arecoline followed by atropine. These well-known drugs are used so that the results may be compared with those obtained by other methods.

cause the animal to make special attempts to breathe deeply, or other convulsions or squirming movements may occur. A sufficient dose of nembutal may control these movements, but in some cases this may cause too great a weakness of the heart or too great a fall in the blood pressure. Such difficulties can usually be overcome by injecting smaller doses of the bronchoconstricting drugs. Possibly section of the nerves to the carotid sinuses and bodies might help in some cases.

Fig. 3 illustrates the action of two common drugs, areeoline and atropine, on the lung and on the blood pressure. There is a certain differenee in form between this tracing and one made by a plethysmographic method. In the latter ease the narrowest part of the lung traeing is usually near the middle of the up and down stroke of the lung tambour, and a general inerease in tone of the whole lung musculature can hardly be seen in the plethysmographic tracing. But with this pulley and lever method even a slight increase in general muscular tone can be seen, even though there may be no apparent constriction of the bronchioles as this is usually interpreted in plethysmographic

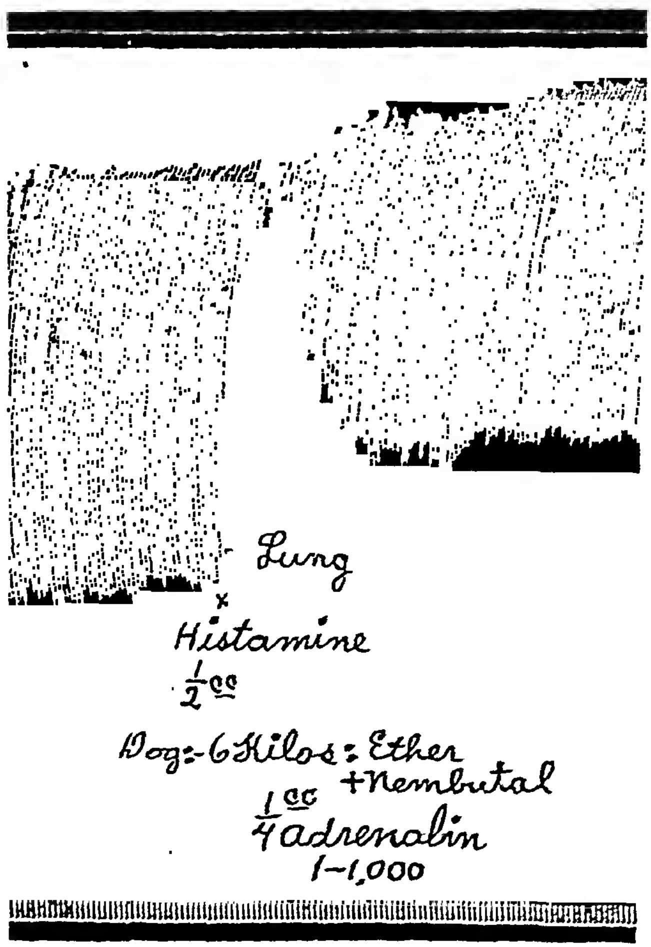


Fig. 4.—Lung tracing showing the action of histamine and adrenalin. Note increased tone (general rise of record) after histamine, even though adrenalin causes partial bronchodilatation.

tracings. A general upward shifting of the whole lung tracing as made by the pulley and lever method means an increase in tone of the whole lung musculature. Probably in the plethysmographic record the changes recorded are due mainly to alterations of the lung musculature in the transverse diameter of the lung lobes, but in the pulley and lever method the changes are obviously mainly in the longitudinal diameter of the lobe.

It can be noted that the upper limit of the left-hand portion of the lung tracing in Fig. 3 shows a kind of saw-tooth effect. This appears to be due to a peristaltic-like waxing and waning of the tone of the lung muscles. While a regular periodic variation in the force with which the air is delivered by the artificial respiration machine might produce such a variation, still as a result of many observations I believe that these effects are generally due to changes occurring within the lungs themselves, or possibly in some instances, due to changes in the pulmonary circulation brought about by variations in the efficiency of the heart.

It is advisable that the air blown into the trachea to inflate the lungs should not be introduced as a sudden strong blast. This may be avoided by passing the air through some closed vessel holding a gallon or more, just before the air is blown into the tracheal cannula. I have frequently used a five-pound ether can for this purpose. Two five-eighth inch tubulatures about one inch long were soldered into the can, one in each end, and this was placed in series with the rubber respiration tube near to the tracheal cannula. The can thus serves as a buffer to tone down a blast of air that may be too strong.

Fig. 4 shows the action of histamine and adrenalin on the lung. It will be noted that the adrenalin partly but not completely overcame the muscular constriction caused by the histamine. The persistent action of the histamine is shown by the gradual rise of the whole tracing, even after the adrenalin has produced a considerable dilatation of the bronchioles. It may be that this progressive increase in tone is due to an action on longitudinal muscle fibers running in the lung lobe more than to an effect on the circular fibers which run in the walls of the bronchioles.

SUMMARY

A simple, practical method for recording lung contractions is presented, together with some results obtained by nerve stimulation and the administration of some common drugs. This method may be useful in ordinary class experiments. It keeps the normal innervation and circulation of the lungs intact.

REFERENCES

1. Jackson, D. E.: Experimental Pharmacology and Materia Medica, ed. 2, St. Louis, 1939, The C. V. Mosby Co.
—: J. Pharmacol. & Exper. Therap. 4: 291, 1913.
—: ibid. 5: 479, 1914.
2. Sollmann, Torald, and Gilbert, A. J.: A Cinematic Study of Bronchiolar Reactions, Proc. Soc. Exper. Biol. & Med. 36: 16, 18, 1937.

A NEW FORM OF MYOCARDIOGRAPH TOGETHER WITH THE TECHNIQUE NECESSARY FOR ITS USE*

D. E. JACKSON, PH.D., M.D., CINCINNATI, OHIO

A VARIETY of special devices for recording contractions of the heart have been used by many investigators in the past.[1] Each of these devices has generally been designed with the object of overcoming one or more of the special difficulties involved in the nature of the experiment. Perhaps the most disturbing feature in the average experiment is the constant up and down or wavering movement which the heart assumes as a result of the inflation and deflation of the lungs by the artificial respiration machine when the chest is opened. The device shown in Fig. 1 has been designed to reduce this disturbance to a minimum. Since the device consists of two parts, the only connection between which is the string (*6*), it is easy to place each part in the most convenient position on the operating table. The writing lever and its mountings may be placed near the drum and at such an angle as will render the recording most efficient.

The experiment is performed by opening the chest of the anesthetized animal by a median longitudinal incision, the sternum being sawed through endwise, after which the chest is opened laterally about three or four inches (dog) and secured in that position by the ligatures which are tied at the sides of the operating board. Care should be taken not to rupture one of the internal mammary arteries in case both should arise from a single stem. The pericardium is opened by a median longitudinal incision, and each side of the membrane is attached up to the chest wall by a single stitch. The pericardium thus forms a kind of hammock in which the heart rests. Care must be taken to see that the heart or the incoming large veins, particularly the inferior vena cava, are not closed off or obstructed by any pull on the pericardium. It may frequently be desirable to make one or more incisions in the cut edges of the pericardium so that the heart may be permitted to beat freely and without any obstruction whatever. The stand carrying the heart levers is now brought near the edge of the operating board and so adjusted that the short levers (*2*) and (*3*) can be placed just over the heart (*1*), the levers being so oriented that their movements will be approximately in the same direction as the longitudinal axis of the heart. By means of the adjustment which is regulated by the setscrew (*5*) the levers are moved near together so that they may be sewed to the heart, each by a single stitch of a very strong thread. ("Button hole" thread is very satisfactory.) Thus the levers are left close together until the recording lever is brought up to the drum. Then, to match the other adjustments, the levers are moved farther apart again and fastened in place by the set screw (*5*). The purpose of this is to put the heart muscle on the proper tension between the attached levers. Oth-

*From the Department of Pharmacology of the University of Cincinnati College of Medicine.
Received for publication, September 16, 1943.

erwise increase or decrease of tone in the heart would not be properly shown by the writing lever.

The string (*6*) should have as little stretch as possible. (A fishing line is very satisfactory.) This string passes through the small coil spring (*9*) over the pulley (*16*), two and one-half inches in diameter, to the pulley (*17*), four inches in diameter, and finally is attached by a bulldog clamp to the writing lever. The bulldog clamp permits ready adjustment of the string so that the lever can be made to write higher or lower on the drum or the magnification can be quickly changed. The spring (*9*) serves as a flexible hollow tube through

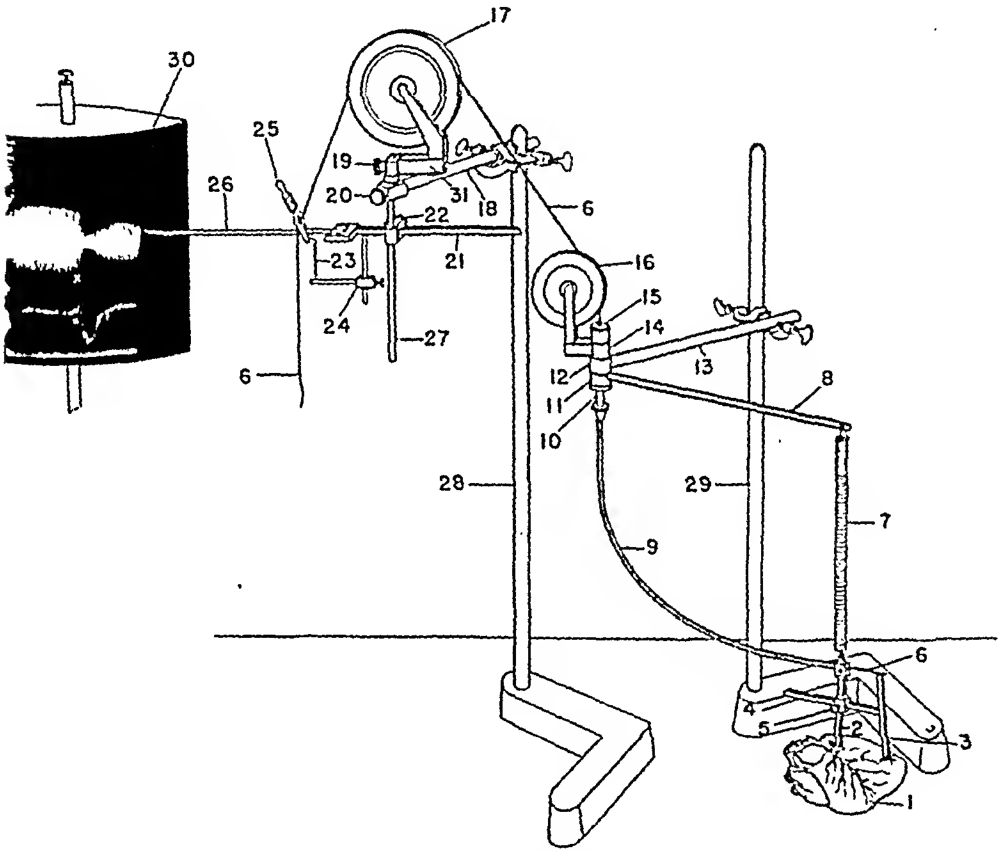


Fig. 1.—A diagrammatic representation of the myocardiograph and of its method of application. The distance between the two stands may be adjusted to suit the size and arrangement of the dog board or operating table, the position of the drum, etc., by simply extending or shortening the segment of the string *6* between the two pulleys. For discussion, see text. Device made by the Max Wocher & Son Company, Cincinnati, Ohio.

which the string slides freely. The spring is made of stiff steel or brass wire and is about one-fourth inch in diameter. The wire is wound closely, and the spring can be bent freely in any direction without changing the length of the enclosed segment of the string. It is this feature which permits the heart to be moved about by the lungs as they are inflated and deflated without these movements being recorded on the drum. The springs which I have used are sold in "dime" stores and are used to hold curtains back against window casings. The springs are cheap and very efficient.

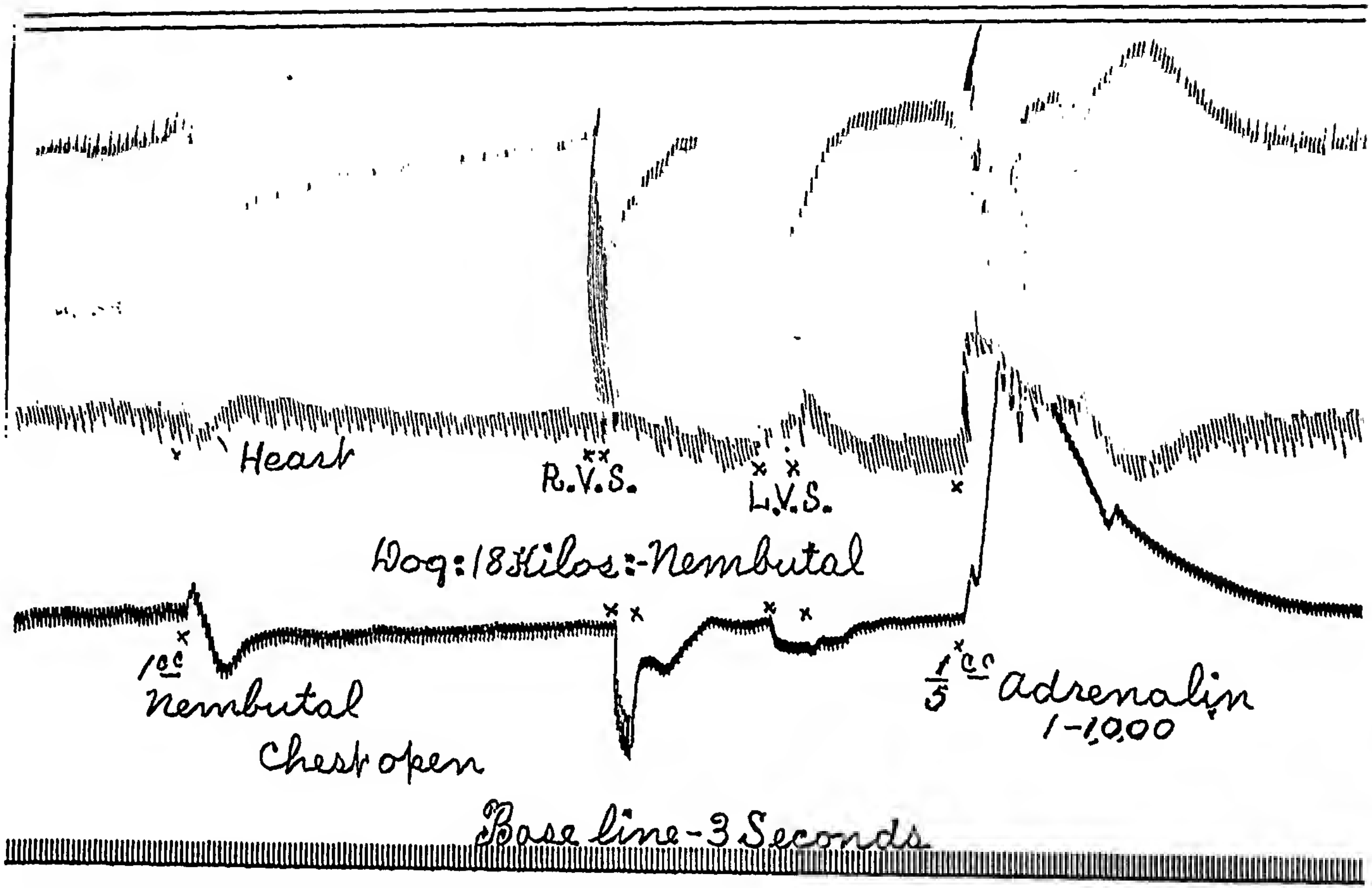


Fig. 2.—Myocardiogram and blood pressure tracing, showing the effects produced by nembutal, vagus stimulation, and injection of adrenalin. For discussion, see text.

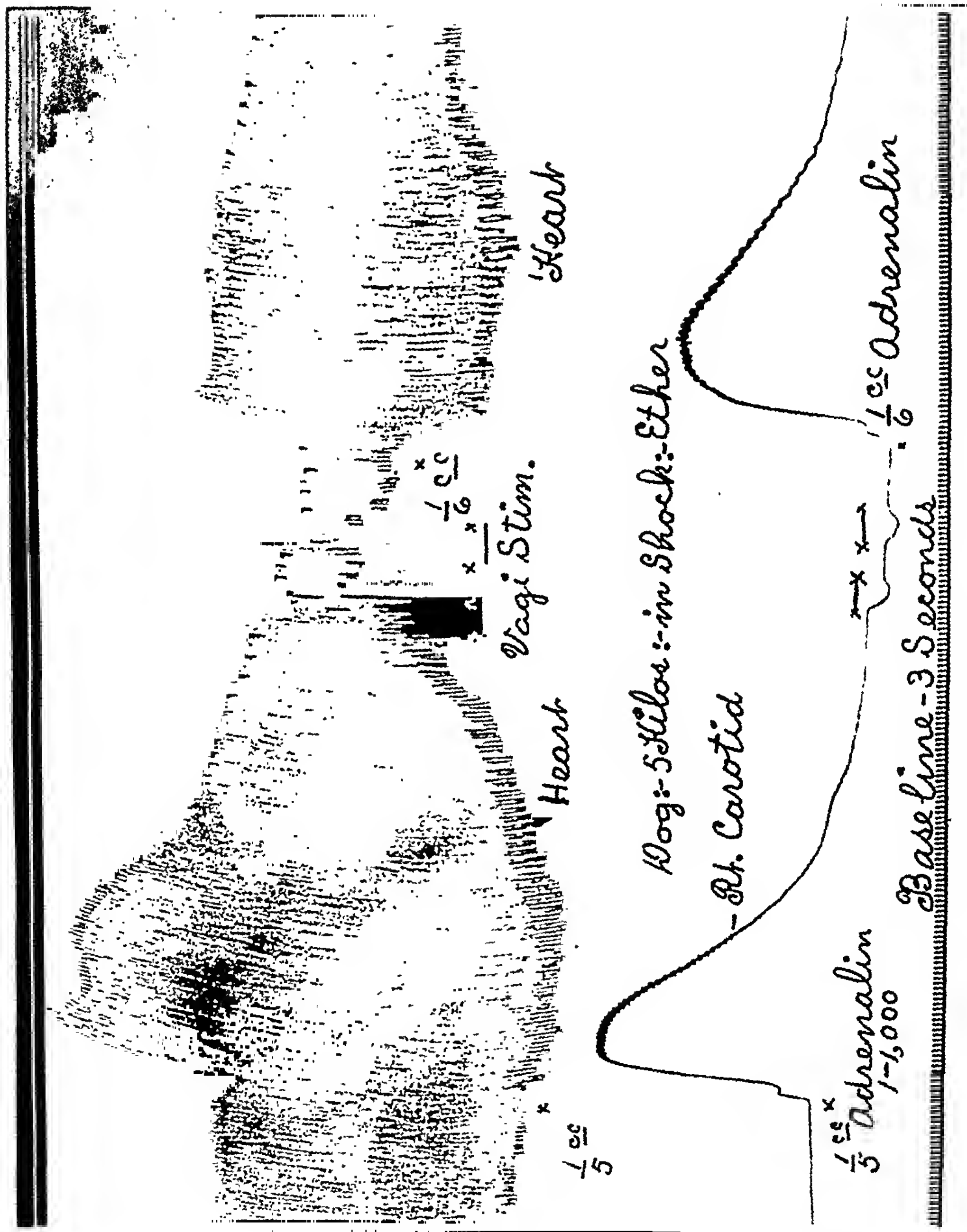


Fig. 3.—Myocardiogram and blood pressure recorded from a dog in deep shock. For discussion, see text.

The spring (*9*) is attached to the metal tube (*10*) which passes upward through the cylindrical parts *11, 12,* and *14.* The rod (*13*) is firmly fastened to the cylinder *12,* and rod *8* is attached to cylinder *11,* while the wood pulley (two and one-half inches in diameter) is attached to cylinder *14.* All of these parts can be firmly fixed in any desired position by merely tightening the nut *15,* which is threaded on to the upper end of tube *10.* The spring *7* supports the heart levers and holds them in position just above and barely touching the heart before they are stitched to the heart. This prevents the instrument from adding any extra strain on to the heart. The pulley *16* turns very freely on its axis,

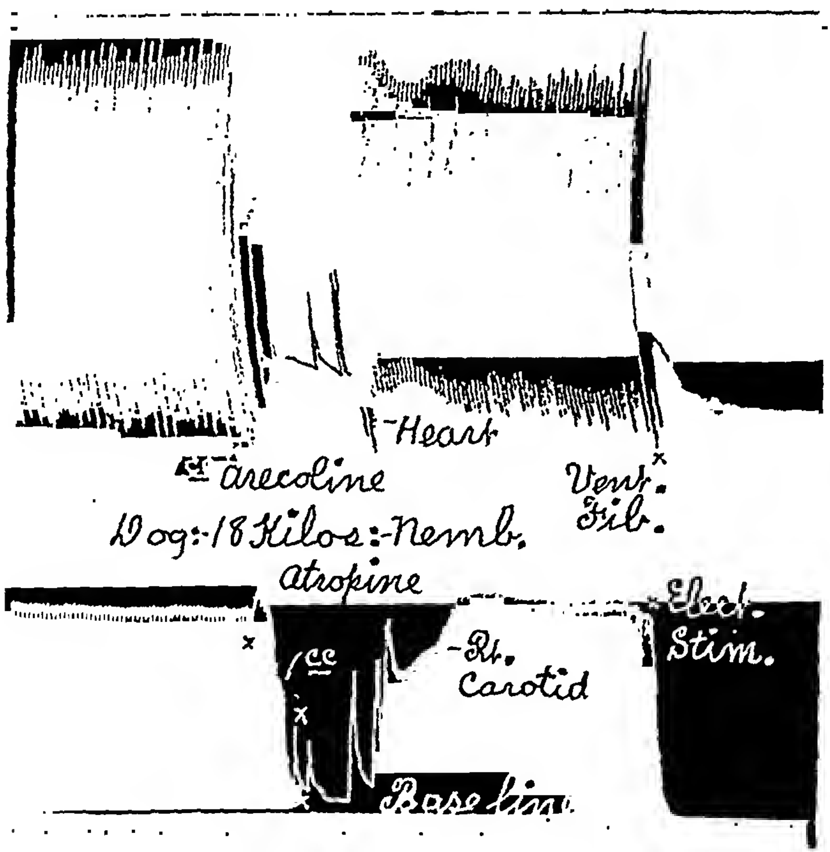


Fig. 4.—Myocardiogram and blood pressure tracing showing the action of arecoline and atropine and the development of ventricular fibrillation. For discussion, see text.

and it can be rotated on the tube *10,* so as to face the larger pulley (*17*) on the recording device. By means of the parts *18, 19, 20, 27,* and *31,* the pulley *17* (made of wood) can be adjusted in six different ways. The writing lever can also be independently adjusted up and down, back and forth, or circularly around the rod *27.* Tension on the writing lever (*26*) is regulated both by the weight of the bulldog clamp and by the coil spring *23* which can be adjusted by means of the setscrew and part *21.* Positive artificial respiration must be used with this machine.

Fig. 2 illustrates some common results recorded by this device. The blood pressure was recorded from the right carotid artery. Nembutal was injected, the right and left (intact) vagi nerves were stimulated, and adrenalin was injected intravenously. The right vagus was considerably more active than the left. It will be noticed that the excursions of the heart were actually considerably reduced when it was acting against the very greatly increased blood pressure caused by the adrenalin, but as the pressure fell, the excursions increased considerably. There is always some chance that a certain amount of "fling" may be recorded by the lever when the heart is beating very fast. The lever should be light and flexible. I have found that very thin spring steel is satisfactory, but perhaps aluminum of the proper thickness and temper might be better.

Fig. 3 shows the myocardiogram and blood pressure of a dog in shock. Two injections of adrenalin were given, and the vagi nerves were stimulated. The blood pressure was so low that the vagus inhibition hardly showed in the blood pressure record, but the effect is obvious in the myocardiogram. Also the brief period during which adrenalin is effective in shock is well shown.

It should not be forgotten that experiments of this character may show a number of points which are not revealed by electrocardiographic tracings, and in many instances observations in one field might well be used to supplement information obtained in the other.

It is sometimes surprising to note how much tension or counterweight must be put on the writing lever in order to secure the optimum contraction records from the heart. The heart tracings should generally be started with an amplitude of about two and one-half to three inches, but it not infrequently happens that a slight stimulation of the heart may cause the record quickly to reach an amplitude of five or more inches, or it may become so marked that the tracings cannot well be recorded on the available drum space. In these cases the height of the initial tracing should be reduced before the stimulating procedure is started. It is highly essential that all parts of the apparatus be properly adjusted before the record is started. A certain amount of experience, and in each case some trial and error, must be carried out in order to secure a maximum of success.

Fig. 4 shows the action of arecoline and atropine on the heart and blood pressure. At the end of the tracing the heart was stimulated directly with a Faradizing current and thrown into fibrillation. The myocardiogram shows the complete diastolic state into which the heart quickly passes as the ventricles begin to fibrillate and the blood pressure promptly falls to zero.

SUMMARY

A new form of myocardiograph is described. Tracings which are made with it and which show the action of some common drugs and of some laboratory procedures are presented, together with a discussion of the technique involved.

REFERENCE

1. Several methods and instruments for recording myocardiographic tracings are given in Jackson's "Experimental Pharmacology and Materia Medica," ed. 2, St. Louis 3, Mo., 1939, C. V. Mosby Co. Most of the operative technique needed for the use of the device described in this paper is given in detail in the above text.

CHEMICAL

THE QUANTITATIVE DETERMINATION OF COMBINED CHOLESTEROL IN THE PRESENCE OF BILE*

Francis F. Foldes, M.D.,† Boston, Mass.

In the course of some experiments it became necessary to determine the eventual combined cholesterol content of the bile of different animals. In looking for a suitable method we found two methods described: one by Wright[1] and the other by Riegel and Rose.[2] The method described by Riegel and Rose appeared to be too complicated for use in a clinical laboratory. The method described by Wright proved to be unsatisfactory in our hands. By adding known quantities of cholesterol esters (acetate propionate and palmitate) alone and mixed with cholesterol to bile, the results we obtained with the Wright method were inconsistent. In the presence of bile, digitonin failed to precipitate free cholesterol quantitatively; furthermore, neither free nor combined cholesterol could be extracted quantitatively with petroleum ether from the residue of the evaporated alcohol-ether extract.

By a simple modification of the method described by Lloyd[3] for the determination of total cholesterol, we were able to determine the combined cholesterol content of bile with satisfactory accuracy. Since the bile of the animals we tested did not contain ester cholesterol, we checked the method by adding known quantities of free and combined cholesterol, dissolved in alcohol, to bile.

METHOD

Bile was diluted ten times with distilled water. To 4 c.c. of this diluted bile, 20 c.c. of 95 per cent alcohol were added in a 50 c.c. Erlenmeyer flask. After mixing thoroughly and adding a piece of quartz to avoid bumping, the mixture was heated in a water bath between 70 and 80° C. for 5 minutes. After heating, the contents of the flask were filtered through a fat-free filter into another 50 c.c. Erlenmeyer flask. The filter was washed twice with 3 c.c. of warm alcohol. Following this, the filtrate was shaken for 2 minutes with 30 c.c. of petroleum ether in a separatory funnel. After the separation of the alcohol and petroleum ether layers, the alcohol layer was allowed to flow back into the flask which had contained the filtrate. The petroleum ether phase was then poured out of the top of the funnel. The last 1 to 2 c.c. of petroleum ether in which there was a small amount of alcohol containing bile was left behind and was subsequently added to the alcohol fraction in the Erlenmeyer

*From the Anesthesia Laboratory of the Harvard Medical School at the Massachusetts General Hospital.

†Resident in Anesthesia, Massachusetts General Hospital, Boston, Mass.

Received for publication, August 7, 1942.

flask through the stopcock. The procedure of extraction was repeated twice more. Because of the great solubility of bile salts and bile pigments in alcohol slightly (1:6) diluted with water and the equally great solubility of cholesterol in petroleum ether, after the third extraction all of the cholesterol was contained in the petroleum ether fraction and was collected in a 100 c.c. volumetric flask. All nonlipid bile constituents were contained in the greenish yellow alcohol fraction. The careful performance of the separation of the two fractions is essential for correct results, because the presence of even small amounts of bile in the petroleum ether fraction can be the cause of gross inaccuracies.

TABLE I

COMPARISON OF THE AMOUNT OF TOTAL AND COMBINED CHOLESTEROL DETERMINED AND CALCULATED

EXP. NO.		TOTAL CHOLESTEROL		COMBINED CHOLESTEROL	
		FOUND MG.	CALCULATED MG.	FOUND MG.	CALCULATED MG.
1.	4 c.c. palmitate solution*	0.35	---	0.22	---
	4 c.c. palmitate solution	0.34	0.35	0.23	0.22
	0.2 c.c. dog bile	0.24	---	0	---
	4 c.c. palmitate sol. and 0.2 c.c. dog bile	0.57	0.58	0.23	0.22
	4 c.c. palmitate sol. and 0.2 c.c. dog bile	0.57	0.58	0.21	0.22
2.	4 c.c. palmitate solution*	0.35	---	0.23	---
	4 c.c. palmitate solution	0.34	0.35	0.23	0.22
	0.2 c.c. cat bile	0.17	---	0	---
	4 c.c. palmitate sol. and 0.2 c.c. cat bile	0.50	0.52	0.22	0.23
	4 c.c. palmitate sol. and 0.2 c.c. cat bile	0.51	0.52	0.21	0.23
3.	0.3 c.c. acetate solution*	1.07	---	0.98	---
	0.3 c.c. acetate sol.	1.09	1.07	0.94	0.98
	0.2 c.c. dog bile	0.25	---	0	---
	0.3 c.c. acetate sol. and 0.2 c.c. dog bile	1.30	1.32	0.96	0.98
	0.3 c.c. acetate sol. and 0.2 c.c. dog bile	1.29	1.32	0.95	0.98
4.	0.3 c.c. acetate solution*	1.07	---	1.03	---
	0.3 c.c. acetate solution	1.07	1.03	1.03	1.03
	0.2 c.c. rabbit bile	0.15	---	0	---
	0.3 c.c. acetate sol. and 0.2 c.c. rabbit bile	1.25	1.22	1.01	1.03
	0.3 c.c. acetate sol. and 0.2 c.c. rabbit bile	1.21	1.22	1.00	1.03

*Determinations marked were made according to Bloor and Knudson's[1] method.

After the separation is completed, the extract is made up with petroleum ether to 100 c.c. Fifty c.c. of this are set aside and are used for the determination of total cholesterol as described by Lloyd.[3] The other 50 c.c., used for the determination of combined cholesterol, are placed in a 50 c.c. beaker or Erlenmeyer flask. (The volumetric flask is washed out with 1 to 2 c.c. of petroleum ether twice.) After adding a piece of quartz to avoid bumping, it is evaporated gently on a steam bath under a hood. The evaporation of petroleum ether, previous to the addition of digitonin, is necessary, because digitonin does not quantitatively precipitate cholesterol dissolved in petroleum ether. The evaporation must be slow; otherwise a large part of the cholesterol

remains on the wall of the vessel. After the evaporation of petroleum ether, 12 to 15 c.c. of hot alcohol-ether mixture (3:1) are added, allowing this to run down slowly on the wall of the vessel, thereby washing down those cholesterol particles which have precipitated on the side of the vessel. To ensure quantitative results, the vessel is now heated on a water bath covered with watch glass for 5 minutes, after which the sides are washed down with 2 to 3 c.c. of ethyl ether. This done, 2 c.c. of a 0.5 per cent digitonin solution are added, and from then on the determination proceeds exactly as given by Bloor and Knudson.[4] In the colorimetric determinations a red filter was used as recommended by Bloor.[5]

RESULTS

To check the accuracy of the method, a known amount of alcoholic solution of a cholesterol and cholesterol palmitate mixture, or a similar amount of cholesterol and cholesterol acetate, was added to bile, the cholesterol content of which had been determined beforehand. Despite the low solubility of cholesterol and cholesterol esters in alcohol, alcoholic solutions were used to ensure complete extraction by the petroleum ether. The cholesterol content of bile and cholesterol mixtures was also determined, and the values found were compared with the ones expected. The results are shown in Table I. For brevity, the cholesterol and cholesterol palmitate and cholesterol and cholesterol acetate mixtures are listed in the table as palmitate and acetate solutions respectively.

SUMMARY

A simple method is described for the simultaneous determination of total and combined cholesterol in the presence of bile.

REFERENCES

1. Wright, A.: Cholesterol and Cholesterol Esters in Dog Bile, J. Exper. Med. 59: 407, 1934.
2. Riegel, C., and Rose, H. J.: Determination of Free and Combined Cholesterol in Bile, J. Biol. Chem. 113: 117, 1936.
3. Lloyd, S. J.: Comparative Concentration of Human Hepatic Bilirubin and Cholesterol by the Gallbladder, Arch. Surg. 41: 1494, 1940.
4. Bloor, W. R., and Knudson, A.: The Separate Determination of Cholesterol and Cholesterol Esters in Small Amounts of Blood, J. Biol. Chem. 27: 107, 1916.
5. Personal communication of Bloor to Wright; see reference No. 1.

BOOK NOTICES

Indigestion, Its Diagnosis and Management, With Special Reference to Diet*

"INDIGESTION" is a word which mystifies physicians just as frequently as medical terms mystify the layman. Rehfuss says that to the layman indigestion represents "the vast category of conditions, both inside and outside the digestive tract, that makes him conscious of his digestion." It is this fact that makes "indigestion" so vague and hard to interpret, though to the patient it may be the outstanding symptom.

Rehfuss examines indigestion from the etiologic point of view, unraveling much of the confusion caused by the unfortunate nature of the word. Sample chapter heads in his book are as follows: "Nervous Indigestion," "Indigestion due to Allergy," "Indigestion Secondary to Infection," "Indigestion due to Gastric Functional Disturbances," "Biliary Tract Type of Indigestion," "The Indigestion of Cardiovascular Disease," "The Indigestion of Old Age." Each chapter considers the diagnosis and treatment of the special type of indigestion and its underlying cause.

Over 150 pages are devoted to the special place of diet in the treatment of gastrointestinal disease. This should be especially useful to the general practitioner, students, and others as a guide for proper management of food intake, which in all disease is of major concern to the patient.

Rehfuss writes in a light, anecdotal style, drawing freely from his own experience in gastroenterology for emphasis of important points. *Indigestion* can be recommended to all as a teaching book and as a reference book for the practical management of patients with gastrointestinal complaints.

Visual Mechanisms†

VISUAL mechanisms was the subject of one of a series of symposia arranged in celebration of the Fiftieth Anniversary of the University of Chicago in 1941. Problems of vision were discussed from the physical, biochemical, physiologic, anatomic, histologic, and psychological points of view. The present volume contains additional contributions which supplement the eight original papers.

The topics include the anatomy and function of the retina and visual cells, the neurophysiology of the optic pathway, the significance of the geniculostriate system, and the originization of the occipital lobe.

There are more general discussions of cerebral organization, energy relations in vision, and a theoretic consideration of sensory acuity.

Four subjects of special interest at the present time are the photochemistry of visual purple, vitamin A, the effect of anoxia on the visual system, and alpha waves in relation to structures involved in vision.

Visual Mechanisms was written by investigators in the advance guard of visual research today. It acts in no sense as a general text in vision. Its purpose is to give an account of the possibilities of the latest research methods.

---

*Indigestion, Its Diagnosis and Management, With Special Reference to Diet. By Martin E. Rehfuss, M.D., Professor of Clinical Medicine, and Sutherland H. Prevost, Lecturer in Therapeutics, Jefferson Medical College, Philadelphia. 556 pages. W. B. Saunders Co., Philadelphia, 1943.

†Biological Symposia. Edited by Jacques Cattell. Vol. VII. Visual Mechanisms. Edited by Heinrich Klüver. The Jacques Cattell Press, Lancaster, Pa., 1942. Pp. viii + 322.

Autonomic Regulations*

THIS book is a very complete review of the nervous and hormonal mechanism which maintains the constancy of the internal environment against the stresses upon the body from the outside. Emphasis is placed on the mutual relationships of the various organs and how changes in the external environment affect the various organ systems of the body. Adjustment reactions of the respiratory and circulatory systems to carbon dioxide, anoxia, asphyxia, hemorrhage, and hypoglycemia receive attention in the first section of the book. The interrelation of the autonomic nervous system with the endocrine system receives attention, with a thorough review of the literature on the nervous regulation of the hormones of the hypothesis and of the sympathetic, adrenal, and vagoinsulin systems. The integration of the autonomic and somatic nervous systems and somatic reactions to hypoglycemia, asphyxia, and anoxia, etc., makes up the third main portion of the book. The author concludes the book with a summary of the results of the physiologic investigations of autonomic reactions and the application of this material to clinical medicine, neurology, and neuropsychiatry. In all, the book is 300 odd pages long.

There has been need for this work for some time. It is a thorough integration of knowledge which heretofore has been spread through many journals over a number of years. It will be a valuable teaching aid for students of physiology and a source book for physiologists and clinicians with a physiologic approach to disease. For the new and growing field of psychosomatic medicine, *Autonomic Regulations* will serve as a guide and orientor to a physiologic approach, which is so important for the proper development in this young branch of Internal Medicine.

Dr. Gellhorn should be praised for his bibliography, which consists of eleven hundred references to the literature of the autonomic and endocrine systems and related subjects.

Biochemistry of the Skin†

MEDICAL students and clinicians would undoubtedly welcome a satisfactory volume dealing with the chemistry of the skin and changes which occur in disease. Dr. Markowitz' book completely fails to meet the need for such a study. The work is sprinkled with erroneous statements and misspelled words. The bibliography is poorly compiled. The inclusion of such subjects as basal metabolism and racial differences in blood, among others, seems hardly justifiable, while, on the other hand, the important and related subject of the chemistry of sweat is only briefly considered. The chapters on the vitamins and dermatoses contain noncritical discussion of subjects such as achromotrichia, the use of massive doses of vitamin D for psoriasis, and the relation of biotin to cancer. References to the author's clinical experience are few and superficial. The reviewer is left puzzled concerning the value the author attaches to the Caspari diet for malignancy, first described in 1929 and included in the appendix of this book.

Polarography‡

SINCE 1922 there has developed a new theory and practice for rapid qualitative and quantitative determinations of most metals and nonmetals as well as of many organic compounds. The method is considered of value in serologic examination for the detection of sulfhydryl containing compounds, unsaturated acids, carbonyl compounds, certain nitrogen

*Autonomic Regulations. Their Significance for Physiology, Psychology and Neuropsychiatry. By Ernst Gellhorn, M.D., Ph.D., Professor of Physiology, College of Medicine, University of Illinois. With 80 Illustrations and Frontispiece. Cloth, $5.50, 375 pages. Interscience Publishers, Inc., New York, 1943.

†Practical Survey of Chemistry and Metabolism of the Skin. By Morris Markowitz, M.D. Cloth, 196 pages, $3.50. The Blakiston Company, Philadelphia, 1942.

‡Polarography. Polarographic Analysis, Voltammetry and Amperometric Titrations. By I. M. Kolthoff, Professor and Head of Division of Analytical Chemistry, University of Minnesota, Minneapolis, Minn., and James J. Lingane, Instructor in Chemistry, University of California, Berkeley, California. First Edition with 141 Illustrations, 510 pages, $6.00. The Appendix contains tables of the Potentials of Common Reference Electrodes and of Half-Wave Potentials of Inorganic Substances. Eight pages can be bought separately, $0.75. Interscience Publishers, Inc., New York, 1941.

derivatives, and in making other biochemical assays. Any substance which can be electroreduced or electrooxidized can be detected and measured. The most desirable concentrations for analysis range between 10^{-4} and 10^{-2} molar, which on the average can be measured with a deviation of 2 per cent.

The analytic results of this new method depend on the interpretation of graphical current-voltage curves; hence the name "voltammetry" seems appropriate, but its founder Jaroslav Heyrovsky has called it *Polarography*. The decomposition potential of a given substance is characteristic of the particular electroreducible or electrooxidizable substance present. The "half-wave potential" is even more characteristic. The latter is recognized as the potential of the electrode in the solution of unknown against an external reference electrode, indicated by the point on a current-voltage curve where the current is equal to one-half of its limiting value. Under best conditions the limiting current is directly proportional to the concentration of the electroreducible substance. Thus, the "half-wave potential" shows the qualitative composition, while the limiting current measures quantitatively the unknown present, the interpretations being made by referring to curves of known substances. Even mixtures can often be assayed.

The authors have written a treatise, theoretical and practical, which compares with their usual high quality work. They have not only digested the information in all of the four hundred odd papers published on the subject up to 1940, but have supplied much information specifically by their own investigations. Due credit has been given to other workers, and sufficient bibliography has been supplied to aid those desiring more complete information. This book of 500 pages, the only important one written in English on Polarography, should be possessed by all interested in employing the methods and by those studying or teaching the subject.

Spectrophotometry in Medicine*

SPECTROPHOTOMETRIC methods are becoming of more and more importance in laboratory medicine; they are especially valuable in research work. This translation of Ludwig Heilmeyer's "Medizinische Spektrophotometrie" by A. Jordan and T. L. Tippell will be found of great value to any one interested either in the theory of spectrophotometry or its clinical application. Since this is primarily a translation of the original German version published in 1932, few references are made to methods developed since that time. The description of instruments, however, has been supplemented so as to bring it up-to-date. The subject matter is divided into 6 topics: (1) theory and selected methods of spectrophotometry; (2) the spectrophotometry of whole blood, of hemoglobin, and of the most important hemoglobin derivatives; (3) the first breakdown products of hemoglobin; (4) the spectrophotometry of blood serum and of normal and pathologic serum pigments; (5) the spectrophotometry of urine, and of the most important normal and pathologic urinary pigments; and (6) the spectrophotometry of bile, ascitic fluid, and cerebrospinal fluid.

*Translation of Ludwig Heilmeyer's "Medizinische Spektrophotometrie" by A. Jordan, M.B., B.S. (Lond.), D.Sc. (Strasbourg), M.R.C.P. (Lond.) Ingénieur-Chimiste E. C. M., Assistant-Pathologist E.M.S.; Jur[illegible] Chemical Pathology to St. Bartholomew's Hospital and T. L. Tippell. Clo[illegible] Adam Hilger Ltd., London, Eng., 1943. Obtainable in U. S. A. from The [illegible], 165 Newbury Street, Boston, Mass.

INDEX TO VOLUME 28

AUTHORS INDEX

In the index following the author's name, the title of the subject is given as it appeared in the JOURNAL.

E

F

G

N

O

P

T

SUBJECT INDEX

Abstracts are indicated by (Abst.) after the page number; book reviews by (B. Rev.) after the page number.

E

F

L

M